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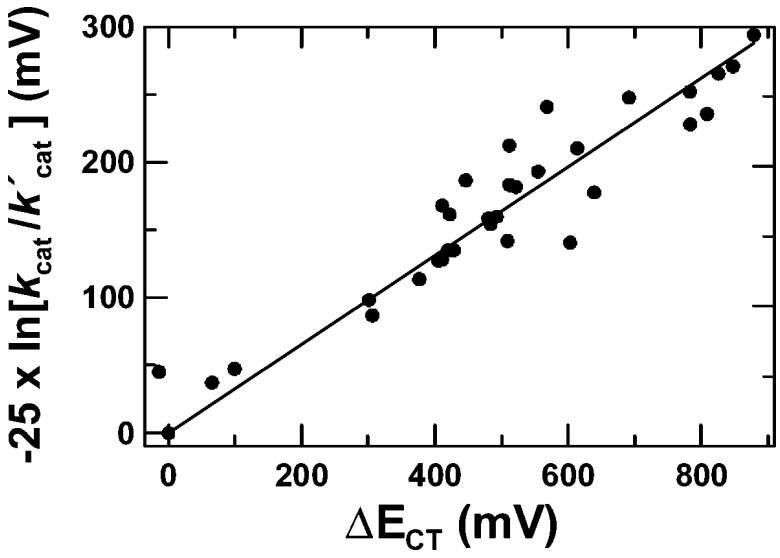

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Relationship between Charge-Transfer Interactions, Redox Potentials, and Catalysis for Different Forms of the Flavoprotein Component of *p*-Cresol Methylhydroxylase

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Abstract: Thirty-three variants of the flavoprotein component of *p*-cresol methylhydroxylase that contain noncovalently or covalently bound flavin adenine dinucleotide (FAD) analogues were studied. A very good correlation was found between the efficiency of *p*-cresol oxidation by these proteins and E_{CT}, the energy for the maximum wavelength for the charge-transfer band of the complex between the bound flavin and 4-bromophenol, a substrate mimic. The correlation covers a range of k_{cat} values that spans over 5 orders of magnitude and values of E_{CT} that span 900 mV, and the analysis of the data provided a value of the transfer coefficient, α, of 0.31. This study demonstrates clearly that the redox properties of both the bound substrate and the flavin cofactor must be taken into account to explain the relative catalytic efficiencies of the variant flavoproteins.

Introduction

What is it about the structure (e.g., the electronic properties) of reactants that allow them to be transformed at a reasonable rate? This question is pertinent to all areas of chemistry. For organic chemists, the question can be addressed by preparing reactant analogues that differ systematically in structure and, ideally, over a wide range in properties. Using each analogue, the reaction is studied when all other reagents and environmental conditions are unchanged. Hopefully, the investigator can tease from the kinetic data information that allows them to assign the importance of various physical properties of the reactants to the process under study. Collectively, such endeavors are known as quantitative structure activity relationship (QSAR)[1] studies.[2]

In addition to solution reactions, there are many examples of QSAR studies for enzyme-catalyzed processes that use modified substrates. However, due to the enzyme's nature (e.g., its steric or electrostatic properties), the types of substituent replacements that can be made often narrow the range of substrates to those with closely similar structures/properties; in other words, there may be a significant collinearity of the physicochemical properties.[2] Furthermore, the substrate is only one component of the reaction. The other component, the enzyme, can have its properties altered by removing a prosthetic group (e.g., a metal ion or organic coenzyme) and replacing it with a cogener, although this may not be easy or even possible. Alternatively, if the structural gene(s) for an enzyme and a convenient gene-expression system are available, a specific amino acyl group of an enzyme can be substituted by another, although the range is limited by the 20 naturally occurring amino acids.

Methods have been developed for incorporating unnatural amino acid into proteins. These included the total chemical synthesis of the protein, semisynthetic methods, the use of chemically modified tRNA molecules for in vitro translation, or, by several genetic manipulations, to significantly alter a microorganism's biosynthetic machinery so that it can incorporate an unnatural amino acyl group into a particular enzyme.[3] Regrettably, these approaches require a great expenditure of time and resources to produce even a single protein variant. Hence, these methodologies are beyond the capabilities of most laboratories for collecting a large family of altered enzymes for a QSAR-type study.

Moreover, it is assumed typically that the properties of enzyme-bound modified substrates or cofactors, or amino acyl side groups, can be predicted from their solution properties. If this were true, one could use the values for numerous variables derived for solution QSAR analyses. However, the environment of an enzyme can perturb the electronic properties of the

(1) Abbreviations: PchF[Y384F] and PchF[R474K], the mutant forms of the flavoprotein where Tyr384 is replaced by Phe, and Arg474 is replaced by Lys, respectively; ^{C}, PchF with covalently bound flavin; 8-Cl-FAD, 8-nor-8-chloro-FAD; 8-Cl-Rbf, 8-nor-8-chlororiboflavin; 5-deaza-FAD, 5-deaza-5-carba-FAD; 5-deaza-Rbf, 5-deaza-5-carbariboflavin; CT, charge transfer; CTC, charge-transfer complex; E^{A}, electron affinity; E_{CT}, energy corresponding to the wavelength for the maximum absorbance of the CTC between protein-bound flavin and 4-bromophenol; $E_{m,7}$ midpoint potentials (NHE) measured at pH 7.0 and 25 °C; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; iso-FAD, 8-nor-6-methyl-FAD; I^{D}, ionization potential; iso-Rbf, isoriboflavin or 8-nor-6-methylriboflavin; MSOX, monomeric sarcosine oxidase; ^{NC}, PchF with noncovalently bound flavin; PCA, pyrrole-2-carboxylate; PchC and PchF, the c-type cytochrome and the flavoprotein subunit, respectively, of *p*-cresol methylhydroxylase; PCMH, *p*-cresol methylhydroxylase; PES, phenazine ethosulfate; Rbf, riboflavin; TS, transition state.

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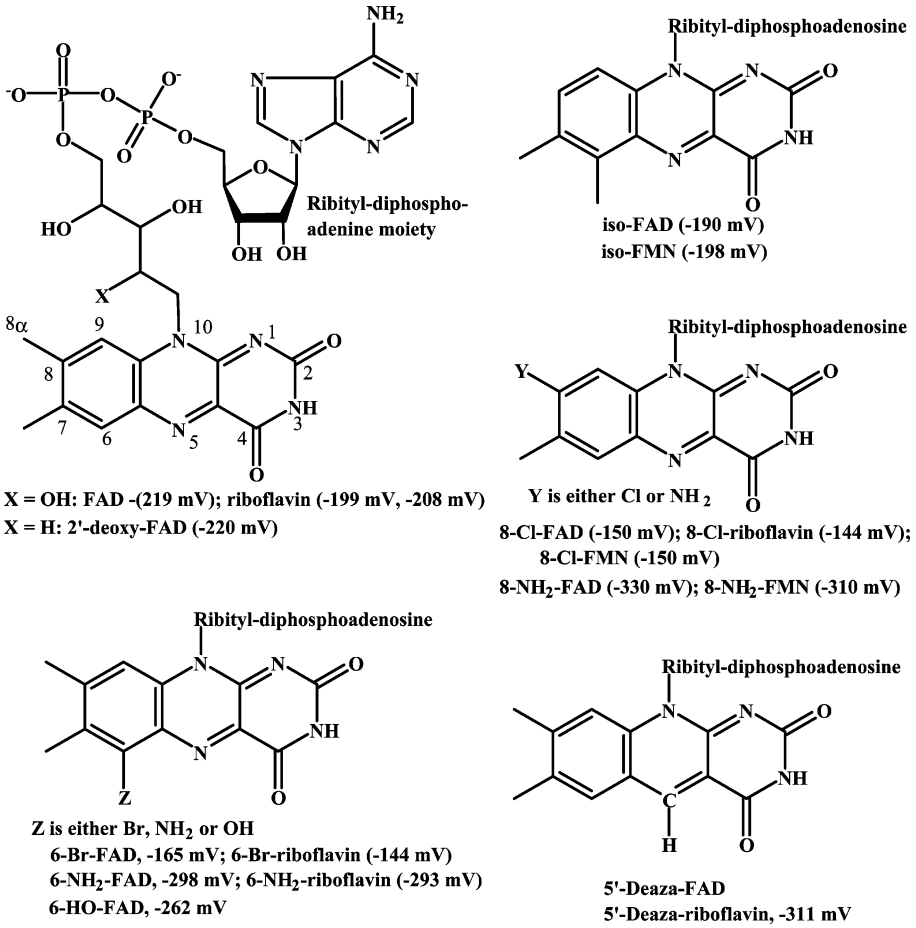

Figure 1. Structures and solution redox potentials ($E_{m,7}$) for the flavins used in the current study. The diphosphoadenine moiety is not present for the riboflavin derivatives, whereas the FMN forms are missing the phosphoadeninyl portion of the structure displayed in the upper left. The potentials are from the following references: Rbf, FAD, 8-Cl-Rbf, 8-Cl-FMN,[6] 2′-deoxy-Rbf,[7] iso-FAD, 6-NH_2-FAD, 6-Br-FAD, 6-HO-FAD,[8] 8-Cl-FAD, 8-NH_2-FAD,[9] 8-NH_2-FMN,[10] iso-FMN, 5-deaza-FMN,[11] 5-deza-Rbf,[6,12] 6-NH_2-Rbf, and 6-Br-Rbf.[6,13]

substrate or cofactor, or alter the amino acyl side group in ways that may be difficult or impossible to predict or quantify, for example, unexpected ionic states, one or more fixed conformations, unusual hydrogen bonds, unusual polarizations, impinging electrostatic fields, charged-transfer interactions, etc.

For QSAR studies involving enzymes, multiparametric equations are required usually to give statistically significant correlations. While a multiparametric (linear) correlation may have predictive value for designing inhibitors or drugs, often a correlation can be complex, confounding, or difficult to grasp in simple conceptual or theoretical terms.

Herein, we describe an enzyme system for which we can measure readily the catalytic power of various altered forms. We have discovered that there is a strong correlation between catalysis and a single, easily measured property of each enzyme variant. This population includes normal and site-specifically altered forms of the protein in isolation or in association with a partner subunit. The variants harbor either normal or modified forms of a cofactor. However, we need not be concerned with the nature of the alteration, as long as we can quantify a particular spectral feature of each protein (vide infra). This feature is a function of the fundamental electronic properties of both the bound substrate and the cofactor.

The enzyme of note is *p*-cresol methylhydroxylase (PCMH, EC 1.17.99.1), a flavocytochrome from *Pseudomonas putida*. This $\alpha_2\beta_2$ dehydrogenase oxidizes *p*-cresol (4-methylphenol) to 4-hydroxybenzyl alcohol, which is oxidized also by the enzyme to 4-hydroxybenzaldehyde. Each α flavoprotein subunit (PchF) of a tightly associated α_2 dimer binds to a *c*-type cytochrome subunit (β or PchC; 9.2 kDa).[4] For each PchF subunit (58.7 kDa), FAD is linked covalently via an ether bond between the 8α-carbon of the flavin's isoalloxazine ring and phenolic oxygen of Tyr384 (Figure 1).[5]

Our *Escherichia coli*-expression system[14] is capable of manufacturing high levels of PchF or site-specifically altered forms thereof. While all of the purified PchF variants are devoid of FAD, each can be reconstituted easily with FAD or one of its analogues. When wild-type PchF with noncovalently bound FAD (PchF{FAD}^{NC})[15] is exposed to PchC, the co-enzyme

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becomes bound covalently to form natural PCMH.[16] As reported herein, this process occurs also for apo-PchF reconstituted with some FAD analogues. When PCMH{F}^{C} (F is FAD or an FAD analogue) is subjected to isoelectric focusing, PchC and PchF with covalently bound flavin (PchF{F}^{C}) separate easily.[17] As a result, for our previous[14,16,18] and current investigations, it has been possible to obtain easily many forms of PCMH{F}^{C}, PCMH{F}^{NC}, PchF{F}^{NC}, and PchF{F}^{C} with very different spectral, catalytic, and redox properties.

To derive a statistically significant global correlation between catalysis and other fundamental properties, such as the redox properties of the flavin and substrate, as presented herein, a large ensemble is required. The proteins used for our investigation have special features that make them ideal for this purpose. All wild-type and mutant forms of PCMH{F}^{C}, PCMH{F}^{NC}, PchF{F}^{NC}, and PchF{F}^{C} are very stable. Due to the high absorptivity, it is very easy to monitor the UV−visible properties of these proteins. The spectra are very sensitive to the redox state of the system, the presence of bound ligands, and the protonation state, the tautomeric state, and substitution of the flavin's isoalloxazine ring system. Finally, all of the proteins retain some catalytic activity, which is easily quantified.

The structures of the flavins employed in the studies are shown in Figure 1. The alterations of the isoalloxazine ring are at the sites that dramatically alter the physical, chemical, redox, spectral and binding properties of the flavin.[20] As described elsewhere,[21] each Rbf derivative was converted to the corresponding FAD form. The spectral properties and redox potentials of the free flavins were measured. In addition, the spectral changes of flavin-reconstituted wild-type and genetically altered form of the flavoproteins and flavocytochromes were recorded for the reductive titrations with sodium dithionite, titrations with the substrates, *p*-cresol and 4-hydroxybenzyl alcohol, titrations with the (nonreducing) product, 4-hydroxybenzylaldehyde, and interactions with the substrate mimic, 4-bromophenol (4-Br-phenol).[14,19,21] For each protein, the catalytic activity for *p*-cresol oxidation was measured by steady-state kinetic assays,[22] and the midpoint redox potential, $E_{m,7}$ (NHE), of the bound flavin was determined.[18,19,21,23] As reported in this paper, the analysis of the results leads to the discovery of a strong correlation between k_{cat} for *p*-cresol oxidation and the frequencies (i.e., E_{CT}, the energy) of maximum absorbance for the charge-transfer (CT) bands for complexes between the proteins and 4-Br-phenol, a substrate mimic that cannot be oxidized by the enzyme.

There are a number of studies demonstrating similar correlations for organic chemical reactions.[24] However, comparable studies with enzymes are scarce because they are more onerous or impossible for the reasons stated earlier. Furthermore, to make any correlation more credible, a large number of unique species with very differ activities and electronic properties are required. The various forms of PchF and PCMH meet the requisite criteria. While previous studies on other enzymes offer guidance,[25] the scope of our findings and the analysis of the data are unprecedented in the biochemical field. Furthermore, unlike nearly all other studies, ours is unusual because we appreciate that not only are the electronic properties of the redox cofactor important, but those of the substrate must also be considered.

For the current treatise, we have used the varied proteins to expand on our earlier thermodynamic studies.[14,18,19] This research represents our attempt to gain a better understanding of the physicochemical basis for redox-enzyme catalysis. We endeavor to analyze and interpret our findings with the same rigor expected for solution reactions. To this end, we have invoked equations derived from valence bond theory,[26] transition-state theory,[27] and Marcus theory.[28]

Experimental Section

Materials. 8-Nor-8-chloro-Rbf (8-Cl-Rbf), 6-bromo-Rbf (6-Br-Rbf), 2′-deoxy-Rbf, 8-nor-amino-RBF (8-NH_2-Rbf), and 5-deaza-5-carba-Rbf (5-deaza-Rbf) were gifts from Professors Peter Hemmerich (deceased) and Sandro Ghisla (University of Konstanz, Germany). 6-Amino-Rbf (6-NH_2-Rbf) and 6-hydroxy-Rbf (6-HO-Rbf) were synthesized as described elsewhere.[13,29] *p*-Cresol, 4-hydroxybenzyl alcohol, 4-hydroxybenzaldehyde, and 4-Br-phenol were purified as reported earlier.[14] The chemicals used and the commercials sources are as follows: sodium dithionite, Kodak Chemical Co.; iso-Rbf (8-nor-6-methyl-Rbf), Merck; disodium 2,6-dichlorophenol indophenol, General Biochemicals, Inc.; glucose oxidase, Miles Laboratories; thionin, Janssen Chimica; toluylene blue, ICN Pharmaceuticals, Inc.; ammonium acetate, International Biotechnologies, Inc.; other reagents, Sigma Chemical Co. or Aldrich Chemical Co.

Protein Purification. The growth of transformed-*E. coli* strains and the protein purification protocol are presented elsewhere.[14,19] PchF,

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PchF[Y384F], and PchF[R434K] prepared by this procedure were completely devoid of *c*-type cytochromes and were FAD-free.

PCMH containing either covalently bound FAD, 6-Br-FAD, 6-NH_2-FAD, 6-HO-FAD, or 2′-deoxy-FAD was processed by isoelectric focusing to completely separate the PchF{F}^{C} subunit from PchC.[17,21] Centricon-30 concentrators (Amicon, Inc) were used to remove the ampholytes from holo-flavoprotein solutions and for buffer exchanges.

Steady-State Kinetic Assays. All kinetic experiments, titrations, and redox potential measurements were done in 25 mM KH_2PO4/KOH buffer, pH 7.0 at 25 °C. Steady-state kinetic assays were carried out by using *p*-cresol as the reducing substrate and phenazine ethosulfate (PES) as the reoxidizing substrate.[22] The reduced PES that formed was monitored spectrophotometrically at 600 nm by its subsequent reduction of 2,6-dichlorophenolindophenol. Kinetic traces were recorded with a Uvikon 933 double-beam spectrophotometer.

Spectroscopy and Titrations. UV−visible spectra were obtained at 25 °C by using a Hewlett-Packard 8453A diode-array spectrophotometer. The family of spectra collected for each titration was analyzed by the SPECFIT program (Factor Analysis software from Spectrum Software Associates, Chapel Hill, NC).

Dithionite titrations, substrate reductive titrations, and redox potential measurements were carried out in deoxygenated buffer under an atmosphere of argon in anaerobic quartz cuvettes.[30] Except for those of *p*-cresol, all solutions were made anaerobic by using several vacuum/O_2-free argon flushing cycles. Because of the volatility of *p*-cresol, its solutions, under argon in a sealed vessel, were rendered anaerobic by the presence of glucose, glucose oxidases, and catalase.[22]

Redox potentials were determined by using the xanthine/xanthine oxidase method described elsewhere.[21,23] This procedure requires the presence of methyl viologen and a reference dye with an appropriate redox potential.

The concentrations of anaerobic dithionite solutions were determined by titrating anaerobic solutions of FAD of known concentrations. Dithionite titrations of the enzyme solutions provided the extinction coefficients for the flavins bound to the various proteins. Because the redox potential of the heme is so high, when the midpoint potentials of the flavin in the various flavocytochromes were determined, the heme was completely reduced.

Graphical data were analyzed by nonlinear or linear regression methods by using either a program described earlier[22] or the commercial program NLREG, version 6.1, advanced (Phillip Sherrod, 6430 Annandale Cove, Brentwood, TN; http://www.nlreg.com/index.htm).

Results and Discussion

PchF and PCMH Reconstituted with 6-HO-FAD, 2′-Deoxy-FAD, or 8-NH_2-FAD. A more thorough description of the synthesis, and spectral, redox, and kinetic properties, of 6-HO-Rbf, and PchF and PCMH reconstituted with 6-HO-FAD will be presented in a forthcoming paper. We found that 6-HO-FAD covalently bound very slowly to PchF{6-HO-FAD}^{NC} when this protein was exposed to PchC. Hence, like the situation for PchF{6-NH_2-FAD},[21] it was possible to gather data for four forms of this protein: PchF{6-HO-FAD}^{NC}, PchF{6-HO-FAD}^{C}, PCMH{6-HO-FAD}^{NC}, and PCMH{6-HO-FAD}^{C}.

Anaerobic titrations with dithionite, *p*-cresol, and 4-hydroxybenzyl alcohol were carried out, and midpoint redox potentials at pH 7.0 and 25 °C ($E_{m,7}$) were determined for most of the forms of holo-PchF and PCMH that were investigated. The spectral and redox properties for many of the FAD derivatives in the free and protein-bound forms are described elsewhere.[14,18,19,21] Because this information for 2′-deoxy-FAD or 8-NH_2-FAD has not been published previously, its presentation follows.

2′-Deoxy-FAD bound very tightly to PchF as did FAD.[18] The absorbance maximum shifted from 447 nm for free 2′-deoxy-FAD to 443 nm for the bound form and underwent a slight increase in absorbance (data not shown). These changes are characteristic of other FAD analogues that we have studied.

As with PchF{FAD}^{NC}, 2′-deoxy-FAD that was bound noncovalently to PchF formed a covalent bond when the protein was exposed to PchC to yield PCMH{2′-deoxy-FAD}^{C}. This process occurred with an apparent first-order rate constant of 0.01 s^{-1}, which is a factor of 2.5 less than the constant for normal PchF{FAD}^{NC}. The values of the steady-state kinetic constants k_{cat} and $K_{p-cresol}$ are 8 s^{-1} and 4.3 μM, respectively. The value for the $^D(k_{cat}/K_{p-cresol})$, the intrinsic primary deuterium kinetic isotope effect, was 8.8 when *p*-cresol and *p*-cresol-α,α,α-d_3 were the substrates. This value is similar to that determined for normal PCMH.[31]

PchF{2′-deoxy-FAD}^{C} was obtained from PCMH{2′-deoxy-FAD}^{C} by isoelectric focusing.[32] A UV−visible spectrum of PchF{2′-deoxy-FAD}^{C} showed some resolution of the vibrational transitions of the 443-nm peak, a feature not seen for PchF{2′-deoxy-FAD}^{NC}. The 379-nm peak of the noncovalently bound flavin shifted to 356 nm for PchF{2′-deoxy-FAD}^{C}. These features are similar to those observed for the proteins harboring unadultered FAD. The redox potential ($E_{m,7}$, NHE)[33] for PchF{2′-deoxy-FAD}^{NC} was found to be −46 mV (NHE, reference dye, indigo tetrasulfonate), and for PchF{2′-deoxy-FAD}^{C} it was +28 mV (reference dye, gallocyanine). In the course of the PchF{2′-deoxy-FAD}^{C} dithionite titration, ∼90% of the anionic flavin radical formed initially, as approximately one-electron equivalent of the reductant was added. The apparently stable radical disappeared as more reductant was added to convert it to the fully reduced form of the flavin (data not shown). This behavior was observed for all high-potential flavins bound to PchF.

The k_{cat} values for PCMH{2′-deoxy-FAD}^{C}, PchF{2′-deoxy-FAD}^{C}, and PchF{2′-deoxy-FAD}^{NC} are 8.0, 0.55, and 0.027 s^{-1}, respectively. As expected, both PchF{2′-deoxy-FAD}^{NC} and PchF{2′-deoxy-FAD}^{C} formed a charge-transfer complex (CTC) with 4-Br-phenol (data not shown). The positions of absorbance maximum of the CT peaks are 590 nm for PchF{2′-deoxy-FAD}^{NC} and 650 nm for PchF{2′-deoxy-FAD}^{C}.

The spectra of 8-NH_2-FAD and PchF{8-NH_2-FAD}^{NC} are provided in Figure 2A, and the dithionite titration of the latter is displayed in Figure 2B. During the titration, after each addition of dithionite, an immediate formation of the anionic radical was observed that disappeared slowly over a 20-min time period as it disproportionated to produce fully oxidized and fully reduced flavin. The addition of an excess of 4-Br-phenol to the oxidized enzyme resulted in a small increase in absorbance in the 540−800 nm region, as expected for a CTC. The peak at 484 nm of uncomplexed PchF{8-NH_2-FAD}^{NC} shifted to 470 nm with a slight increase in absorbance for the complex (data not shown).

Charge-Transfer Complexes between 4-Br-Phenol and Various Forms of PCMH and PchF. The crystal structure of the PCMH·*p*-cresol complex shows a $\pi-\pi$ CT-type interaction

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(33) All reported redox potentials, $E_{m,7}$, are in units of millivolts, relative to the normal hydrogen electrode (NHE).

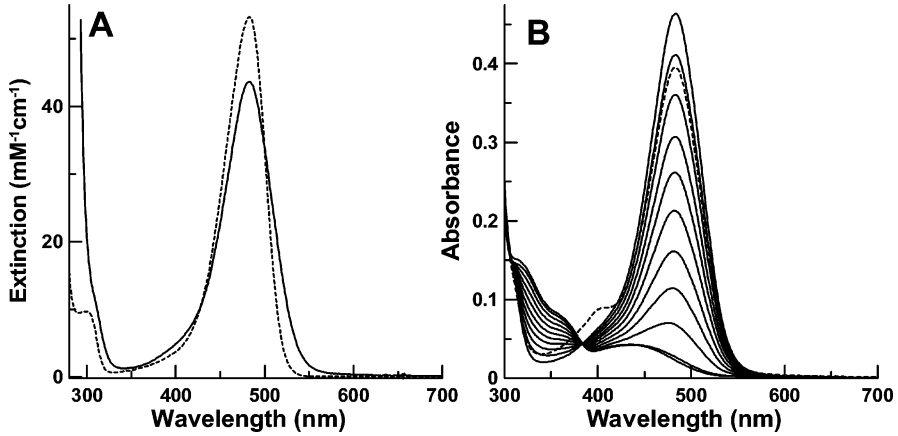


Figure 2. Spectra of 8-NH_2-FAD and PchF{8-NH_2-FAD}^{NC} (A), and the anaerobic dithionite titration of the latter (B). (A) The spectra were recorded for these substances in 50 mM K_2HPO_4/KOH buffer, pH 7.0, at 25 °C. The solid line is the spectrum for PchF{8-NH_2-FAD}^{NC}, and the dashed line is the spectrum for 8-NH_2-FAD. (B) This frame shows the spectral changes recorded 20 min after adding 0, 1.17, 2.34, 3.50, 4.66, 5.86, 6.97, 8.30, 9.29, 10.44, and 11.58 μM dithionite (cumulative concentrations) to 10.5 μM PchF{8-NH_2-FAD}^{NC} in 50 mM K_2HPO_4/KOH buffer, pH 7.0, at 25 °C. The dashed-line spectrum was recorded immediately after the 1.17 μM dithionite addition. The peak at 400 nm indicates the formation of the anionic flavin radical, which disappears after 20 min.

between the substrate and the pyrazinoid and pyrimidinoid portions of the flavin's isoalloxazine ring system.[4] To shed more light on this aspect, 4-Br-phenol was selected as a substrate mimic. Because it lacks an alkyl moiety para to the hydroxyl group, this phenol cannot be oxidized by PCMH.[34] The size of the bromo group is approximately the same as a methyl group, although the electronic properties of 4-Br-phenol are quite different from those of *p*-cresol. Using UV−visible spectroscopy, in previous work[14,18,19,21] and for the experiments reported herein, it has been shown that 4-Br-phenol forms CTCs with normal and site-specifically altered forms of PchF and PCMH reconstituted with FAD and derivatives thereof. Although the positions of the maxima shift, the shape of the CT band remains relatively unchanged from one protein to another; the widths are similar, and the extinction coefficients of the absorption maxima are about 2 mM^{-1} cm^{-1}.

Because only the three-dimensional structure of the PCMH/*p*-cresol complex is available,[4] it was assumed that the stereochemistries of binding of the *p*-cresol and 4-Br-phenol are essentially the same; each should hydrogen bond via its phenolate oxygen to the phenolic hydroxyl groups of Tyr95 and Tyr473, and both would have stacking interactions of their π-systems with that of flavin's isoalloxazine ring. Furthermore, in the active site, on the side distal to the isoalloxazine ring, the benzene rings of *p*-cresol and 4-Br-phenol should be boxed in tightly by the hydrophobic portions of Trp284, Trp394, Ile429, and Val438. Hence, it is not surprising that both phenols show similar binding constants for the studied proteins, that is, 5−25 μM.

According to valence bond theory,[26] the transition energy E_{CT} for the absorbance maximum of a CT band is defined by the equation:

$$E_{CT} = h\nu_{CT} = I^D - E^A - W \qquad (1)$$

For this equation, I^D is the ionization potential of the donor, E^A is the electron affinity of the acceptor, and $\nu_{CT} = c/\lambda_{max}$, where c is the speed of light. W, the work term, which is usually small in value,[26a] is a function of the "no bond" interaction as well as the Coulombic attraction energy of the CTC. It was assumed that E^A can be equated with the redox potential for the formation of the flavin semiquinone, E_1, or the two-electron midpoint redox potential, $E_{m,7}$.[11,24a] It was assumed also that the redox potential for bound 4-bromphenol provided a good estimate for its I^D value.[35]

The maximal wavelengths for 4-Br-phenol CT bands and the $E_{m,7}$ values for these have been determined for 33 forms of PchF and PCMH in the current and published studies (Table 1).[14,18,19,21] Table 1 lists also the steady-state k_{cat} values for the oxidation of *p*-cresol, when PES was used as the reoxidizing substrate.

A case can be made that k_{cat}/K_M is a better measure of the catalytic efficiency of an enzyme because it is generally a simpler function of intrinsic rate constants than is k_{cat}. However, the *p*-cresol K_M values for different forms of PchF and PCMH vary from about 5 to 25 μM, whereas the k_{cat} values vary by over 5 orders of magnitude from 0.00096 to 120 min^{-1}. Hence, using k_{cat}/K_M values will not impact significantly the results and conclusions derived from our investigations.

The Correlation between E_{CT} for 4-Br-Phenol CTCs and Catalytic Activity. Inspection of Table 1 reveals interesting trends for the energies of the CT interactions of 4-Br-phenol with the variant forms of PchF and PCMH and their rates of *p*-cresol oxidation. For example, there seems to be a correlation between E_{CT} and $E_{m,7}$ for the bound FAD. However, a plot of ΔE_{CT} versus $\Delta E_{m,7}$ shows curvature and a fair amount of scatter (Figure 3A). (ΔE_{CT} and $\Delta E_{m,7}$ values were determined using the E_{CT} and $E_{m,7}$ values for PCMH{FAD}^{C} as the references.) Because E_{CT} is a function of I^D and E^A (both defining one-electron processes), perhaps using E_1 (the one-electron potential) values, instead of two-electron potentials, $E_{m,7}$, would improve the correlation (vide infra).

Another factor that likely contributes to the trend seen in Figure 3A results from using $E_{m,7}$ values for flavins in the

(34) (a) McIntire, W. S.; Hopper, D. J.; Singer, T. P. *Biochem. J.* **1985**, *228*, 325−335. (b) McIntire, W. S.; Bohmont, C. In *Flavins and Flavoproteins*; Edmondson, D. E., McCormick, D. B., Eds.; Walter de Gruyter: Berlin and New York, 1987; pp 677−686.

(35) Massey, V.; Meah, Y. M.; Xu, D.; Brown, B. J. In *Flavins and Flavoproteins*; Ghisla, S., Kroneck, P., Macheroux, P., Sund, H., Eds.; Rudolf Weber, Agency for Scientific Publications: Berlin, 1999; pp 645−653.

Table 1

protein[a]	λ_{CT} (nm)	k_{cat} (s^{-1})	$E_{m,7}$ (mV)	$\Delta E_{m,7} \approx \Delta E^A$ (mV)	E_{CT} (mV)	ΔE_{CT} (mV)	$-25.7 \times \ln(k_{cat,X}/k_{cat,PCMH})$ (mV)	$\Delta I^D = \Delta E_{CT} - \Delta E_{m,7}$ (mV)
PCMH{FAD}^{C}	833	121	+87	0	1490	0	0	0
PCMH[Y384F]{8-Cl-FAD}^{NC}	798	27.4	+103	16	1556	65	38.17	82
PCMH{2′-deoxy-FAD}^{C}	841	20.0	+87	0	1476	−14	46.26	−14
PCMH{8-Cl-FAD}^{NC}	781	18.3	+90	3	1589	99	48.54	102
PCMH[Y384F]{FAD}^{NC}	698	3.8	+48	−39	1796	306	88.12	267
PCMH[R474K]{FAD}^{C}	693	2.4	+82	−5	1791	301	100.7	296
PCMH{6-Br-FAD}^{C}	665	1.3	+63	−24	1867	376	116.5	353
PchF{FAD}^{C}	655	0.75	+62	−25	1895	405	130.6	380
PchF[Y384F]{8-Cl-FAD}^{NC}	653	0.73	+58	−29	1901	411	131.4	382
PchF{2′-deoxy-FAD}^{C}	650	0.55	+28	−59	1910	420	138.6	361
PCMH{iso-FAD}^{NC}	647	0.55	+31	−56	1919	429	138.6	373
PCMH[Y384F]{5-deaza-FAD}^{NC}	593	0.44	+19	−68	2093	603	144.3	535
PCMH{5-deaza-FAD}^{NC}	621	0.42	+19	−68	1999	509	145.6	441
PchF[R474K]{FAD}^{C}	629	0.26	+30	−57	1973	483	158.3	426
PchF[Y384F]{FAD}^{NC}	630	0.22	+2	−85	1970	480	162.7	395
PCMH{6-NH2-FAD}^{C}	626	0.21	−174	−261	1983	493	164.0	232
PchF{8-Cl-FAD}^{NC}	649	0.19	+15	−72	1912	422	165.7	350
PCMH{6-HO-FAD}^{C}	653	0.15	ND	ND	1901	411	172.7	ND
PchF{6-NH_2-FAD}^{C}	583	0.10	−252	−339	2129	639	182.5	300
PCMH{6-NH_2-FAD}^{NC}	617	0.085	−187	−274	2012	522	186.6	248
PchF{FAD}^{NC}	620	0.080	−16	−103	2002	512	188.1	409
PchF{6-Br-FAD}^{C}	641	0.070	−48	−135	1937	446	191.6	312
PCMH{6-HO-FAD}^{NC}	607	0.054	−87	−174	2045	555	198.3	468
PchF{2′-deoxy-FAD}^{NC}	590	0.027	−46	−133	2104	614	216.0	481
PCMH{8-NH_2-FAD}^{NC}	620	0.025	−244	−331	2002	512	218.0	181
PchF{6-NH_2-FAD}^{NC}	546	0.013	−275	−362	2273	783	234.0	421
PchF{6-HO-FAD}^{C}	540	0.0098	−130	−217	2299	809	242.1	592
PchF{iso-FAD}^{NC}	603	0.0080	−102	−189	2059	568	247.3	379
PchF{8-NH_2-FAD}^{NC}	569	0.0061	−283	−370	2182	691	254.3	321
PchF[R474K]{FAD}^{NC}	546	0.0051	−106	−193	2273	783	259.0	590
PchF{5-deaza-FAD}^{NC}	536	0.0030	−300	−387	2316	826	272.5	439
PchF{6-HO-FAD}^{NC}	531	0.0024	−208	−295	2338	848	278.3	553
PchF[Y384F]{5-deaza-FAD}^{NC}	524	0.00096	−300	−387	2369	879	302.2	492
PchF{6-Br-FAD}^{NC}	ND	ND	−128	−215	ND	ND	ND	ND

[a] For the *italicized* proteins, the data are from the present study. The data for the other proteins are from other published studies.[14,18,19] $\Delta E_{m,7} = E_{m,7}$(protein) − $E_{m,7}$(PCMH). $\Delta E_{CT} = E_{CT}$(protein) − E_{CT}(PCMH). X represents the various proteins. ND indicates not determined.

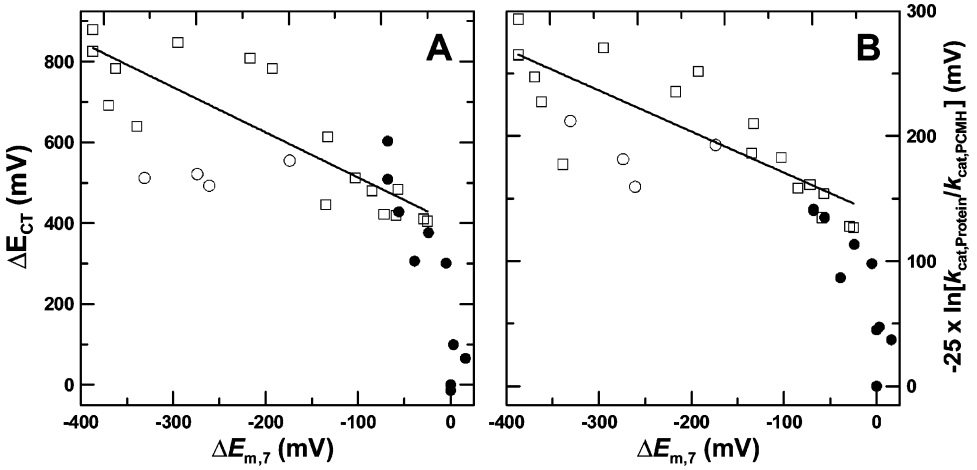


Figure 3. (A) Plot of ΔE_{CT} versus $\Delta E_{m,7}$. $\Delta E_{CT} = E_{CT}$(protein) − E_{CT}(PCMH), and $\Delta E_{m,7} = E_{m,7}$(protein) − $E_{m,7}$(PCMH). The □ symbols are the data for the various forms of PchF, and the ● and ○ symbols are the data for the different forms of PCMH. The ○ symbols represent the data points for PCMH-{6-NH_2-FAD}^{C}, PCMH{6-NH_2-FAD}^{NC}, PCMH{8-NH_2-FAD}^{NC}, and PCMH{6-HO-FAD}^{NC}. The straight line is the linear regression fit to the points represented by the open squares; slope = −1.12 ± 0.16, intercept = 398 ± 38 mV, and $R^2 = 0.726$. (B) Plot of $-25 \times \ln(k_{cat,protein}/k_{cat,PCMH})$ versus $\Delta E_{m,7}$. The □ symbols are the data for the various forms of PchF, and the ● symbols are the data for the different forms of PCMH. As in frame A, the ○ symbols represent the data points for PCMH{6-NH_2-FAD}^{C}, PCMH{6-NH_2-FAD}^{NC}, PCMH{8-NH_2-FAD}^{NC}, and PCMH{6-HO-FAD}^{NC}. The straight line is the linear regression fit to the points represented by the □ symbols; slope = $-\alpha$ = −0.328 ± 0.056, intercept = 138 ± 13 mV, and $R^2 = 0.658$.

flavocytochromes that were measured when the heme was fully reduced, whereas the E_{CT} values are derived from the interaction of 4-Br-phenol with the flavins in PCMH forms harboring oxidized heme. Thus, the $E_{m,7}$ values for the bound flavins when the heme is oxidized may differ from those listed in Table 1. It is impossible to measure the flavin $E_{m,7}$ values when the heme is oxidized because the latter's potential is always much greater than that of the former. For the present study, it was assumed that the values for $E_{m,7}$ for bound flavin are not that different when the heme is oxidized or reduced. However, the charge of the heme's iron changes from 3+ to 2+ on its reduction. Hence, the different electrostatic fields emanating from the proximal

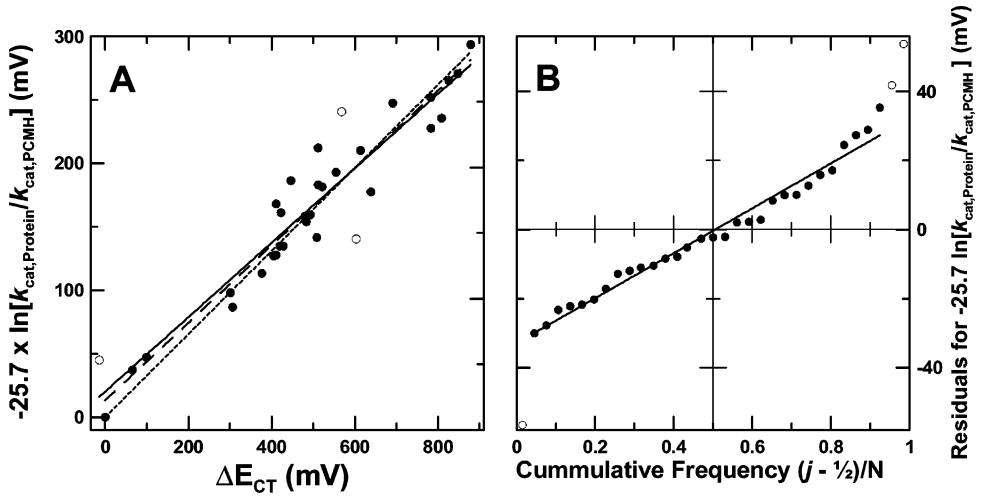


Figure 4. (A) Plot of $-25.7 \times \ln(k_{cat,protein}/k_{cat,PCMH})$ versus ΔE_{CT}. The ○ symbols are the data points for PCMH{2′-deoxy-FAD}^{C}, PchF{iso-FAD}^{NC}, and PCMH[Y384F]{5-deaza-FAD}^{NC}. The solid line results from linear-regression analyses of all of the points (slope = $\alpha = 0.306 \pm 0.018$, intercept = 20.7 ± 9.8 mV, $R^2 = 0.894$). The long-dashed line is the linear-regression fit using the points represented by the ● symbols only; slope = $\alpha = 0.313 \pm 0.016$, intercept = 13.8 ± 8.7 mV, and $R^2 = 0.932$. The short-dashed line is the linear-regression fit when the line is forced through the origin; slope = $\alpha = 0.340 \pm 0.017$, intercept = 0 mV, and $R^2 = 0.924$. (B) Cumulative frequency plot of the $-25.7 \times \ln(k_{cat,protein}/k_{cat,PCMH})$ residuals for the solid-line fit to the data in frame A. After the residuals are ordered from the most negative value to the most positive, each is assigned a number $j = 1, 2, 3, ...N$, where N is the total number of points (33, in this case). For each ranked point, the cumulative frequency is calculated from the equation $(j - 1/2)/N$.[36] The plot is constructed by plotting the residuals versus the cumulative frequency. The line is the linear regression fit of the ● point, which excludes the outliers (○).

heme,[4] which impinges on the flavin, may alter one- and two-electron $E_{m,7}$ values of FAD (i.e., anionic FAD$^{\bullet -}$ and FADH$^-$, respectively, are formed) in PchF·PchC$_{red}$ relative to those for PchF·PchC$_{ox}$. We suspect that the $E_{m,7}$ value for FAD or its analogues would be somewhat larger than those provided in Table 1 for the PCMH in the Fe(III) state.

When we consider only the data for the PchC-free PchF forms (□ in Figure 3A), there is a rough linear correlation for the plot of ΔE_{CT} [$= \Delta(I^D - E^A) = \Delta I^D - \Delta E^A$] versus $\Delta E_{m,7}$. The slope of the line for a linear regression fit of these points, 1.12 ± 0.16 ($R^2 = 0.726$), is close to a value of 1, which should be the case when $\Delta I^D \approx 0$ and $\Delta E^A \approx \Delta E_{m,7}$. The y-intercept for the line is +399 ± 38 mV, the same as the 380 mV value calculated earlier for the difference between the 4-Br-Phenol I^D values for PchF^C and PCMH.[19] Some of the difference may be attributed to a suspected higher $E_{m,7}$(FAD) value for PCMH with oxidized heme (vide supra). However, realistically, this cannot come close to accounting for the entire 380−400 mV difference.

By presuming that $\Delta E_{CT} = \Delta(I^D - E^A)_{4-Br-phenol} \approx \Delta(I^D - E^A)_{p-cresol}$ (vide infra), and because $\Delta E_{m,7}$ ($=\Delta E^A$) for FAD is assumed to be constant, then $\Delta I^D_{4-Br-phenol} \approx I^D_{p-cresol}$. Thus, for *p*-cresol oxidization, we conclude that $I^D_{p-cresol}$ for PchF is greater than $I^D_{p-cresol}$ for PCMH by as much as 380 mV. In other words, it is much more difficult to remove an electron from the highest occupied molecular orbital (HOMO) of *p*-cresol when it is bound to free PchF^C than when it is bound to PchF^C in PCMH. If one assumes the typical value for the electron-transfer symmetry factor, $\alpha = 0.5$ (see eq 2), then the transition-state free energy for the reaction is decreased by 0.5 × 380 mV = 190 mV (8.8 kcal/mol), which is reasonable for an efficient catalyst.

Because the $E_{m,7}$(flavin) values for the various forms of PCMH with oxidized heme are unknown, it might be expected also that the change in the oxidative power, as reflected in the k_{cat} values for *p*-cresol oxidation, would not be correlated well with the midpoint potentials. The plot in Figure 3B shows this to be the case. Because we assume that $\Delta I^D \approx 0$ and $\Delta E^A \approx \Delta E_{m,7}$ for the data for the PchC-free forms of PchF, then the slope of the line for these proteins provides an estimate of α (vide infra and eq 2) and is equal to 0.33 ± 0.06 ($R^2 = 0.648$).

Interestingly, the plots displayed in Figure 3A and B show similar trends. This suggested that there might be a better correlation between $\Delta\ln(k_{cat})$ and ΔE_{CT}. Significantly, correlating k_{cat} and E_{CT} eliminates the need for true $E_{m,7}$(FAD) (or E_1) values for the PCMH variants when the heme is oxidized, or, for that matter, for any of the PchF or PCMH forms. Figure 4A displays the plot of $\Delta\ln(k_{cat})$ versus ΔE_{CT} for 33 forms of PCMH/PchF. Even though there are three somewhat aberrant points (those for PchF{iso-FAD}^{NC}, PCMH{2′-deoxy-FAD}^{C}, and PchF[Y384F]{5-deaza-FAD}^{NC}), a linear correlation is apparent.

When the subunits associate, the total effect on catalysis is a result of changes in $E_{m,7}$ for both the flavin (ΔE^A) and the substrate (ΔI^D). With the k_{cat} value for PCMH{FAD}^{C} as the reference, the k_{cat} value for any other form of PCMH or PchF can be calculated from the following equation:[27a]

$$k_{cat,Protein}/k_{cat,PCMH} = \exp(\alpha \times [\Delta E^A - \Delta I^D]\mathcal{F}/RT) = \exp(-\alpha \times \Delta E_{CT}\mathcal{F}/RT) \quad (2)$$

As before, ΔE_{CT} is obtained from the shift of the absorbance maxima for CTCs with 4-Br-phenol, and $k_{cat,PCMH}$ is the catalytic constant for normal PCMH. It was assumed also that $(\Delta E^A - \Delta I^D)_{p-cresol} \approx (\Delta E^A - \Delta I^D)_{4-Br-phenol}$, as is the case for the binding of different phenols to OYE.[11] By taking the natural logarithm of both sides of eq 2, and using $\mathcal{F}/RT = 25.7$ mV ($\mathcal{F}$ is Faradays constant, R is the gas constant, and T is the temperature = 25 °C), then plotting $-25.7 \times \ln(k_{cat,Protein}/k_{cat,PCMH})$ (mV) against ΔE_{CT} (mV) should produce a straight line with a slope, α. This is the plot shown in Figure 4A. When three aberrant points (○ in Figure 4A) are ignored, the correlation coefficient is $R^2 = 0.932$ for the linear regression fit of the data, $\alpha = 0.313 \pm 0.016$, and the intercept is 13.8 ± 8.7 mV (for the regression analysis, each point is given equal

weighting = 1; when the line is forced through the origin, $R^2 = 0.924$ and $\alpha = 0.340 \pm 0.017$). It is important to point out that the linear correlation holds for a ΔE_{CT} range of 900 mV, and over 5 orders of magnitude in k_{cat}. In addition, the linearity supports the proposition that the k_{cat} values truly reflect the intrinsic catalytic power of each protein.

We believe that the scatter of the points in Figure 4A is due, in part, to the inherent errors in the k_{cat} values, which are typically 5−10%. Also contributing to the inaccuracy of k_{cat} values are the errors in estimating the extinction coefficients for the various proteins from anaerobic standardized-dithionite titrations, which result in errors in the protein concentrations. As any biochemist appreciates, there is always some variability in properties of a particular enzyme from one isolation to another. While we did not detect any problem using different batches of enzyme, this could be a contributing factor. The scatter may diminished also by using the values of the specificity constant, k_{cat}/K_M, rather than those of k_{cat} (vide supra), although we believe that this will not impact the correlation significantly. Finally, estimating the λ_{max} for CT peaks was done by eye, and by taking the first and second derivatives of the spectra. However, due to the low absorbance of these peaks and interference from the heme absorbance for the PCMH forms, determining these values accurately was difficult; occasionally, the absorbance of PchF-free PchC was subtracted from the absorbance of a PCMH form to get a better estimate.[21] We estimate the error to be about 5−10 nm. Attempts to use a spectral curve fitting program to get better values failed. Because of the low absorbance, small shifts of the baseline, and the noise, the charge-transfer bands typically were best fit by two or more peaks.

The residuals for the $-25.7 \times \ln[k_{cat,Protein}/k_{cat,PCMH}]$ versus E_{CT} fit (the solid line in Figure 4A) were analyzed by constructing a cumulative frequency plot.[36] If the errors for the data point are random and distributed normally, then this plot should define a straight line that intercepts residual = 0 mV when the cumulative frequency is 0.5. The cumulative frequency plot (Figure 4B) does show the correct behavior, when the outliers are ignored; outliers are those points that deviate from the straight line. This plot is characteristic for real data with normally distributed random errors,[36] and it provides an easy way to identify outliers. We believe that this analysis provides compelling evidence that the linear correlation displayed in Figure 4A is valid and highlights the need for a large population of protein species. For all similar biochemical studies reported in the literature, far fewer points were used. If there is scatter, with one or two aberrant points (outliers) in a smaller population, the result could deviate significantly from the correct one.

The straight line in Figure 4A, with slope $\alpha = 0.31$, that nearly intersects the origin, seems to lend credence to our analysis and underlying assumptions. However, we remain cautious concerning the true value of α, if $(\Delta E^A - \Delta I^D)_{p-cresol} = C \times (\Delta E^A - \Delta I^D)_{4-Br-phenol}$, where C is a constant. While this would not alter the linear correlation seen in Figure 4A, the true value of α for *p*-cresol oxidation would be somewhat different.

The parameter α, known as the symmetry factor or the transfer coefficient,[27a] is defined as

$$\alpha = d(\Delta G^{\ddagger})/d(\Delta G^{\circ}) = d[\ln(k)]/d[\ln(K)] \quad (3)$$

where $\Delta G^{\ddagger}$ is the change in the free energy between a reactant and its transition state (TS) (the activation energy), and ΔG° is the change in free energy between reactants and its products.[27] The parameter α is equivalent also to the exponent in the Brønsted equation, $k = AK_{eq}^{\alpha}$.[27a] Originally, α was thought to provide a measure of the extent of the reaction; α = 0, 1, and 0.5 for the reactant state, the product state, and a symmetrical TS, respectively. However, it has been found that values of α do not necessarily related to transition-state structure.[28a−c]

The Brønsted α can be recast in terms of Marcus theory with the following equation:

$$\alpha = {}^1/_2 + \Delta G^{\circ}/(2\lambda) \quad (4)$$

where λ is the reorganizational energy of the system.[28a] The parameter α varies from 0 to 1 over the range of ΔG° as $|\Delta G^{\circ}| \rightarrow \lambda$. If the reorganizational energy is large or as $\Delta G^{\circ} \rightarrow 0$, then α = 0.5.

With Marcus theory as a basis, Kreevoy and co-workers derived the following expression:[28b]

$$\alpha = \chi \pm (\tau - 1)/2 \quad (5)$$

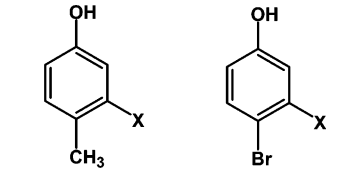
For this equation, $\chi = {}^1/_2 + \Delta G^{\circ}/(2\lambda)$, where χ is equal to $d(\Delta G^{\ddagger})/d(\Delta G^{\circ})$ at constant λ.[28b] Note that the parameter χ has the meaning ascribed originally to α (see eq 4), the traditional Leffler−Hammond parameter. Equation 5 reduces to eq 4 when $\tau = 1$. For eq 5, the plus sign is invoked when the acceptor is varied with a constant donor, and the minus sign is used for the converse situation. Because of this change in sign, the value of α will only be the same for these two cases when $\tau = 1$.

The "tightness factor", τ, varies in value from 0 to 2; a value of 0 indicates the in-flight hydrogen is completely detached from the donor and acceptor, and a value of 2 indicated full bonding to both the donor and the acceptor, that is, a hypervalent hydrogen. If the bond order is conserved, τ would equal 1.[28d] This parameter provides a measure of the "displacement of the critical configuration toward structures that do not resemble either reactant or product, which is required to describe charge distribution and reactive bond lengths". The "critical configuration" is the "most probable one for crossing the hardest-to-attain-dividing surface separating products and reactants."[28b]

Clearly, for us to extract any useful information from the value of α, we must first have measures of ΔG° and λ. Hypothetically, if the reorganizational energy were large, then $\chi = {}^1/_2$. Because α = 0.31, then $\tau = 0.62$. (Using these values of χ and τ, α would be equal to 0.69 for the situation where the donor is varied with constant acceptor.) With $\chi = 0.5$, the critical configuration is halfway between those of the reactant and product. However, this value for τ would indicate a rather narrow spatial separation between the hydride donor (methyl group of *p*-cresol) and acceptor (*N*5-position of the flavin), and the transferred hydrogen takes on a partial negative charge during transfer. The charge on the in-flight hydrogen, $\delta = \tau - 1$,[28e] would be −0.38, if our initial assumption about the magnitude of λ were correct. In the case where $|\Delta G^{\circ}| \approx \lambda$, $\chi = 0$ (assuming ΔG° has a negative value), then $\tau = 0.41$, and $\delta = -0.59$.

(36) Daniel, C.; Wood, F. S. *Fitting Equations to Data: Computer Analysis of Multifactor Data*, 2nd ed.; John Wiley & Sons: New York, 1980; Chapter 3, pp 19−49.

A study complementary to the one presented here involves measuring the rate of substrate oxidation as a function of varied donor (i.e., substrate) and constant acceptor (a particular FAD analog). If eq 4 were applicable, α should be the same whether the acceptor is varied and the donor is constant or vice versa. If eq 5 were applicable, the values of α derived from these two sets of experiments would be different.[28b−c] Using the approach we have developed for PCMH, we would need good mimics for each varied substrate to obtain reliable estimates of E_{CT}. For example, we could use the substrates and corresponding mimic with the basic structures:



ortho -substituted *p* -cresol *ortho* -substituted 4-bromophenol

For these compounds, X could be any number of substituents; prior studies have demonstrated that PCMH is a fairly promiscuous enzyme in that it can oxidize a wide range of substituted 4-alkylphenols.[31,34] If the two complementary studies provide dissimilar values of α (i.e., study 1 where the hydrogen donor is constant and the acceptor varies, and study 2 where the donor varies but the acceptor is constant), we can use both forms of eq 5:[28b]

$$\alpha_1 = \chi + (\tau - 1)/2$$

$$\alpha_2 = \chi - (\tau - 1)/2$$

Because we now have two equations and two unknowns, we can solve for the values of τ and χ:

$$\tau = \alpha_1 - \alpha_2 + 1$$

$$\chi = (\alpha_1 + \alpha_2)/2$$

Is the Formation of a CTC Essential for Catalysis? The potential importance of CT interaction in flavoprotein catalysis has been recognized for quite some time as exemplified by a seminal review on this topic presented by Massey and Ghisla three decades ago.[37] The review exposes several flavoenzymes where a CTC was observed. However, in most of the cases, the complexes were seen when an enzyme reacted with substrate analogues, or when an enzyme converted a substrate analogue, in a nonredox reaction, into a species that formed a CTC with the flavin. In some cases, the CTC was between reduce flavin (donor) and oxidized substrate (acceptor). Furthermore, there are numerous examples where substrate analogues (i.e., inhibitors) bind to a flavoenzyme and perturb the flavin's spectrum, but do not produce the board, long-wavelength absorbance characteristic of a CTC. As these authors pointed out, "...the frequent occurrence of CT absorption bands might be merely an accompanying phenomenon that is dictated by the chemical characteristics of the two partners in the complex." However, these authors point out also "that the energy involved in such molecular complex interactions is of the order of only a few thousand calories per mole, and that the donor/acceptor interaction, per se, contributes to a minor extent to the overall stability of the complex." Therefore, " it is not hard to imagine that such an increased stability could have a very important function in the regulation or direction of a catalytic reaction." For example, for lactate mono-oxygenase, the reactivity of O_2 with the reduced flavin/pyruvate CTC is 200-times faster than that with uncomplexed reduced flavin.[37]

According to Mulliken,[26a,b,38] the wave functions for the ground (G) and excited (Ex) (adiabatic) states for CT (donor/acceptor) complexes are defined as $\Psi_G = a\psi(D,A) + b\psi(D^+A^-)$ and $\Psi_{Ex} = b^*\psi(D,A) - a^*(D^+A^-)$. $\psi(D,A)$ and $\psi(D^+A^-)$ are the diabatic wave functions, and $a \approx a^*$, $b \approx b^*$, and $a \gg b$ for a weak complex. Thus, a 4-Br-phenol/FAD CTC becomes apparent only when light is absorbed to bring it from the G state (GS) to the Ex state (ExS), which is accompanied by a transfer of electron density from the donor to the acceptor to give the dative state, D^+,A^-. However, for highly active forms of PCMH or PchF, their interaction with *p*-cresol results in a GS adiabatic electron transfer (ET) to yield oxidized substrate and reduced FAD. Therefore, catalysis (GS-ET) and CTC formation (ExS-electron [density] transfer) may be mutually exclusive, and a *p*-cresol/FAD CTC never forms.

Rather than saying that CT interactions, per se, are or are not important to coupled hydrogen/electron-transfer reactions, perhaps it is more accurate to say instead that it is the energies of the LUMO of the acceptor (flavin) and the HOMO of the donor (substrate) that are important. In the case of a nonoxizable substrate mimic, the LUMO and HOMO energies in the enzyme−mimic complex give rise to CT bands, whereas for the substrate, these energies dictate the ease by which coupled hydrogen-electron (e.g., hydride) transfers occur in the enzyme−substrate complex. Therefore, a formal CTC need not form when substrate binds.

For some of our poor enzyme catalyst used in the present study (e.g., $PchF^{NC}$),[14] a substrate-CT absorbance band was seen. This suggests that a complex forms, which displays CT properties, and also undergoes GS electron transfer. This type of mechanism was proposed for a nonenzymic electrophilic aromatic substitution reaction.[24b] Alternatively, the CTC converts slowly to an intermediate that can undergo coupled hydrogen/electron transfer from the substrate to flavin.

Relevant Studies with Other Enzymes. A linear correlation was found between the redox potentials of FAD bound and the natural log of the rate constants, that is, ln(*k*), for NADPH oxidation by oxidized FAD, or for O_2 reduction by reduced FAD for different forms of mercuric ion reductase.[25a] If it is assumed that I^D (for NADPH) is constant for the first reaction, and E^A (for O_2) is constant for the second reaction, then, according to eq 2, there should be a linear correlation. Thus, for NADPH oxidation, $\Delta I^D \approx 0$ and $E^A \approx E_{m,7}$(FAD), but for O_2 reduction, $\Delta E^A \approx 0$ and $I^D \approx E_{m,7}$(FAD). Because of the small number of points and scatter in a plot of $[-25.7\ \text{mV} \times \ln(k/k_o)]$ versus $\Delta E_{m,7}$ (eq 2), it is not possible to get a good estimate of α from these data.

A study of the reduction of *p*-hydroxybenzoate hydroxylase reconstituted with several 8-substituted 8-nor-FAD analogues by reduced *N*-methylnicotinamide provide better data.[25b] From a plot of $[-25.7\ \text{mV} \times \ln(k/k_o)]$ versus $\Delta E_{m,7}$, we estimate that

(37) Massy, V.; Ghisla, S. *N.Y. Acad. Sci.* **1974**, *227*, 446−465.

(38) Mulliken, R. S. *J. Am. Chem. Soc.* **1952**, *74*, 811−824.

$\alpha = 0.53$ ($R^2 = 0.829$). However, a better correlation ($R^2 = 0.958$) was obtained with the equation, -25.7 mV $\times \ln(k/k_o) = A \times \Delta E_{m,7} + B \times \kappa$, where κ represents constants for the 8-substituents of 8-nor-FAD that are based on the size and hydrophobicity of the proton donor and acceptor in CTCs.[39] For this correlative fit, $A = 0.36$ ($= \alpha$?) and $B = -117$ mV. In this case, it appears that ΔI^D for the substrate is constant when bound to each enzyme species.

The rate constant for NADH reduction of 2-methyl-3-hydroxypyridine-5-carboxylic acid (MHPC) oxidase increases as the $E_{m,7}$ values of bound FAD analogues increase.[25c] However, the ln(k) versus $E_{m,7}$ ($= E^A$) plot was smoothly curved, not linear, which suggests that the ionization potentials (I^D) of the substrate also vary when bound to the different forms of the enzyme.

Like PCMH, bacterial monomeric sarcosine oxidase (MSOX) is a flavoprotein containing covalently bound FAD (i.e., 8α-*S*-cysteinyl-FAD). For the normal and the H269N mutant forms of MSOX, the redox potentials for the first electron transfer, E_1, that is, for flavin radical formation, are essentially the same at 79.5 and 76 mV, respectively.[25d] However, the reductive half-reaction with L-proline had "limiting rates" (k_{lim}) = 7.4 and 0.198 min^{-1}, respectively, although the K_D values for ES complexes of both proteins were very similar. Titrations with the substrate analogue and competitive inhibitor, pyrrole-2-carboxylate (PCA), showed the formation of CTCs with the maximum of absorbance at 608 nm for wild-type MSOX and 566 nm for the H269A mutant. This translates into a value of -152 mV for $\Delta(E^A - I^D)_{PCA}$. Using eq 2 and assuming $\alpha = 0.5$, a value of -181 mV is obtained for $\Delta(E^A - I^D)_{L-proline}$. If it is assumed that the values of E_1 are the same whether PCA or L-proline are bound or not to the wild-type and mutant protein, then $\Delta E_1 \approx \Delta E^A \approx 0$. Therefore, $\Delta I^D_{PCA} \approx 150$ mV and $\Delta I^D_{L-proline} \approx 180$ mV. It should be noted that, unlike the *p*-cresol/4-Br-phenol pair, L-proline and PCA are not isosteric. In fact, because the ring of L-proline is completely saturated, it would not be possible to form the same type of CTC as does PCA.[25d] Thus, it would not be expected necessarily that $\Delta(E^A - I^D)_{PCA} \approx \Delta(E^A - I^D)_{L-proline}$. While the authors of the MSOX study recognize a relationship between the catalytic rates and the energy of CTCs, they did not provide a theoretical foundation for the phenomenon.[25d]

Closing Remarks

It is somewhat surprising that there is such a good linear correlation between $\Delta\ln[k_{cat}]$ and ΔE_{CT} over such a wide range. It might be expected that the binding of the various FAD forms would differ for PchF^{NC}, PchF^{C}, and PCMH, and, therefore, the stereochemistry of bound 4-Br-phenol and *p*-cresol relative to the flavin ring might be somewhat different for each protein. In addition, the position and orientation of catalytic groups and the isoalloxazine ring in the active site may be different for the various types of PchF. This would lead to changes in the rate constant for *p*-cresol oxidation in addition to those resulting from changes in E^A and/or I^D. For example, there may be interactions between a bulky group at the 6-position of the isoalloxazine ring of FAD (Figure 1) with neighboring amino acyl residues of the proteins that are required for catalysis; for example, Glu380, which has a side-chain carboxylate oxygen 3.25 Å from the C-6 carbon of the flavin, is hydrogen bonded to a water molecule that is thought to hydroxylate the *p*-quinone methide intermediate that forms when the substrate is oxidized.[4] However, as long as *p*-cresol and 4-Br-phenol bind to any particular protein with similar stereochemistries, any changes on going from one protein to another should influence $(\Delta E^A - \Delta I^D)_{p-cresol}$ and $(\Delta E^A - \Delta I^D)_{4-Br-phenol}$ in a similar manner. Hence, a linear relationship between ΔE_{CT} and $\ln(k_{cat,Protein}/k_{cat,PCMH})$ would be maintained. It is possible that minor variations in the stereochemistry for binding of 4-Br-phenol relative to that for *p*-cresol could lead to a slight inequality for $(\Delta E^A - \Delta I^D)_{p-cresol}$ and $(\Delta E^A - \Delta I^D)_{4-Br-phenol}$ for a particular protein, and this contributes to the scatter of points seen in Figure 4A.

For enzyme kinetics, a parameter such as α cannot be determined experimentally as a function of an applied potential as can be done for electrode-based electrochemical studies.[40] Furthermore, attempts to correlate catalytic efficiency and the potentials of the enzyme-bound redox catalyst (e.g., a coenzyme such as flavin) often failed, because the polarization of the substrate was not taken into account. In fact, it is usually impossible to do so. By measuring instead the change in the energies of the CTCs between PchF/PCMH and a nonreacting substrate analogue (vide supra), one can effectively introduce such a potential scale. Hence, this allows us to invoke existing theories and approaches. The observed constancy of α over a wide range of E_{CT} values indicates that the constructed enzyme "potential" scale is equivalent to the electrochemical potential scale.

Stated in a different manner, as long as a CTC forms and it can be detected by the appearance of a CT band, there is no need to measure the one- or two-electron redox potentials for a bound cofactor or substrate. This is of particular importance for enzyme systems where determining these potentials is cumbersome or impossible. However, in our case, measuring the $E_{m,7}$ values for the bound flavins was straightforward. By assuming that these values provide accurate estimates of the E^A values, the values of I^D for each form of enzyme can be estimated from the E_{CT} values by using eq 1. Inspection of the ΔI^D values (Table 1) reasserts an important outcome of our work, which is the need to take into account the contribution of the electronic properties of the bound substrate (HOMO energies/ionization potentials/redox potentials) in addition to those of the cofactor (LUMO energies/electron affinities/redox potentials) to adequately predict catalytic efficiency. Apparently, both the E^A of the flavin and the I^D of the substrate are tuned when bound to PCMH so as to make this oxidoreductase a more efficient catalyst.

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