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Investigation of the Pathway for Inter-Copper Electron Transfer in Peptidylglycine α-Amidating Monooxygenase

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Dopamine β-monooxygenase (DβM) and peptidylglycine α-amidating monooxygenase (PAM) belong to a small family of homologous copper proteins that are localized to neurosecretory vesicles and catalyze the production of neurohormones and neurotransmitters.[1−3] The X-ray structure for the hydroxylating domain of PAM, referred to as peptidylglycine α-hydroxylating monooxygenase (PHM), indicates two copper centers that are separated by a distance of 10−11 Å. The protein structure changes little upon the binding of a peptide analogue and, overall, lacks a hinge motif that would allow the copper centers to approach one another during catalysis[4,5] (Figure 1).

It has been established that both copper centers in PAM and DβM cycle between their +1 and +2 valence states during catalysis, necessitating an electron transfer from the copper site that functions in electron storage (generally referred to as Cu_H or Cu_A) to the site that binds O_2 and substrate, producing hydroxylated product (Cu_M or Cu_B).[6,7] A number of mechanisms have been proposed for the long-range electron transfer between Cu_H and Cu_M that include: (i) the use of a one-electron reduced oxygen species (superoxide ion) as an electron conduit from Cu_H to Cu_M;[8] (ii) the involvement of a protein network, located between the two copper-binding domains, as the pathway for intermetal electron transfer;[5] and (iii) the participation of substrate itself in the electron-transfer process.[5,9,16]

A recent study of the relationship between O_2 consumption and substrate hydroxylation in DβM indicates complete coupling for these processes using substrates that vary in reactivity by ca. 3 orders of magnitude.[10] This rules out a mechanism in which free superoxide ion is used as a courier for electron transfer from Cu_H to Cu_M. Similarly, using site specific mutagenesis to investigate the role of an interdomain, hydrogen-bonded protein network, Eipper and co-workers have recently shown that mutation of a key residue (Q170A) in PAM gives rise to very little change in enzyme activity, ruling out this pathway for electron transfer. These authors propose that the substrate may undergo a translocation from the Cu_M site (Figure 1) to the cavity between the copper centers, as a means of facilitating the reaction.[9]

In the present study, we have examined the hypothesis that the peptide backbone of substrate may provide a pathway for interdomain electron transfer. Two substrates that differ greatly with regard to steric bulk, hydrophobicity, and most importantly length (**1** and **2** below) were synthesized (with protium or deuterium in the α-position of the C-terminally extended glycine) and characterized kinetically, where L_2 is either protium or deuterium:[11]

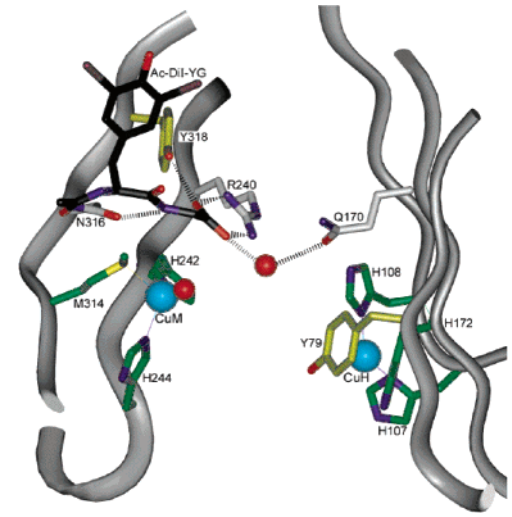
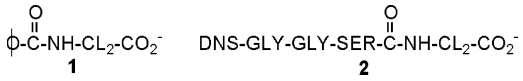


Figure 1. Interdomain structure of PHM with *N*-acetyl-diiodo-tyrosyl-glycine (Ac-Dil-YG) bound close to Cu_M. Modified from ref 5.



Modeling of these substrates into the active site by complexation of the C-terminal carboxylate to Cu_H shows that **2**, but not **1**, is able to bridge the distance from the Cu_H to the Cu_M site. In the case of **1**, the amide carbonyl of the C-terminal glycine is at a distance of ca. 6 Å from Cu_H, requiring that solvent water complete the pathway for electron transfer.

Data were collected under conditions where the substrate binds to the enzyme prior to molecular O_2, with a difference between **1** and **2** being the rate of loss of substrate from the E−S complex in relation to the binding O_2 (rapid in the case of **1** and slow for **2**).[13]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{-}S + O_2 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{-}S\text{-}O_2 \xrightarrow{k_3} E\text{-}P \xrightarrow{k_4} E + P \qquad (1)$$

The key kinetic parameters obtained by measuring rates as a function of the O_2 concentration are $k_{cat}/K_m(O_2)$ (obtained in the limit of low O_2 and saturating substrate, which measures all steps from the binding of O_2 up through the irreversible C−H bond cleavage step) and k_{cat} (at saturating substrate and O_2, representing the chemical step and all steps that lead to the regeneration of free enzyme). Limiting kinetic parameters with a protonated and deuterated extended peptide such as **2** have not been previously available and allow an evaluation of the kinetic isotope effects and rate-

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Table 1. Comparison of Kinetic Parameters for Truncated and Extended Peptide Substrates with PAM[a]

substrate	$k_{cat}/K_m(O_2)$[b] (μM^{-1}, s^{-1})	$^D[k_{cat}/K_m(O_2)]$	k_{cat}[c] (s^{-1})	$^Dk_{cat}$
1[d]	0.14 (0.01)	3.1 (0.3)	37 (0.1)	1.0 (0.1)
2	0.23 (0.03)	2.3 (0.2)	17 (0.8)	1.0 (0.1)

[a] Data collected at pH 6, 37 °C by following the decrease of oxygen concentration using a YSI model 5300 biological oxygen monitor. Reaction mixtures contained 100 mM MES, 30 mM KCl, 10 mM ascorbate, 10 μg/ml catalase, 1 μM $CuSO_4$, and varying levels of O_2 and peptide. Enzyme is a type A rat medullary carcinoma PAM expressed in Chinese hamster ovary cells. [b] Extrapolated to saturating concentrations of substrate. [c] Extrapolated to saturating concentrations of substrate and O_2. [d] From ref 14.

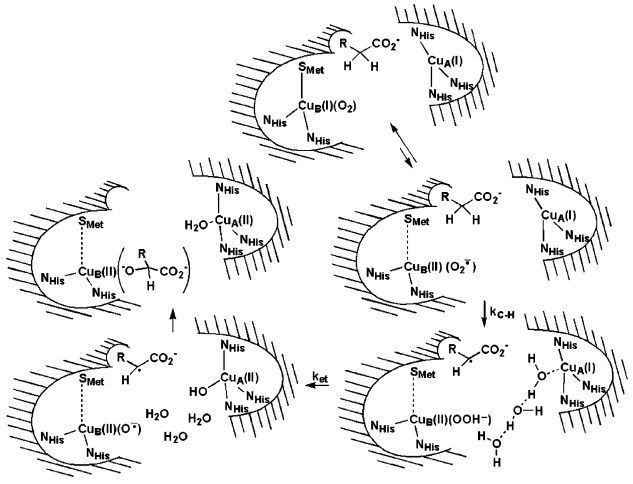
limiting steps for comparison to **1**. As shown in Table 1, $k_{cat}/K_m(O_2)$ is increased less than 2-fold for the extended peptide substrate **2**, and this is accompanied by a small decrease in the measured deuterium isotope effect. It appears that for **2** the overall hydroxylation process is slightly increased relative to O_2 binding but that the magnitude of k_3 is similar for the truncated and extended peptides. In the case of k_{cat}, substrate **2** is about 2-fold slower and there is no measurable KIE with either substrate. The absence of a KIE on k_{cat} indicates that C−H abstraction is fast relative to other rate-limiting steps that may include interdomain electron transfer as well as product release.

Two reaction mechanisms can be advanced for DβM and PAM, in which the interdomain electron transfer takes place either before or after the C−H abstraction step. In the former case, electron-transfer contributes to $k_{cat}/K_m(O_2)$, and in the latter case, it is contained in k_{cat}. The close similarity of the primary parameters and their isotope effects for the enormously different substrate structures of **1** and **2**, leads us to conclude that *the extended backbone of naturally occurring peptide substrates is unlikely to constitute a pathway for full or partially rate-limiting, long-range electron transfer*.

In principle, electron transfer may be sufficiently fast that it does not contribute to the measured kinetic parameters. From the isotope effect on k_{cat}/K_m (Table 1), this parameter contains a significant contribution from C−H cleavage [ca. 10^3 s^{-1}],[14] indicating that electron transfer would need to be much larger than 10^3 s^{-1} to be a "silent" contributor to the k_{cat}/K_m measured for **1** and **2**. Rate constants for electron transfer through proteins over a distance of 11 Å, in the limit where the driving force ($\Delta G°$) and reorganization energy (λ) cancel, have been estimated to range from 10^9 to 10^{10} s^{-1};[15] this may be reduced by 3−4 orders of magnitude in the presence of sizable $\Delta G°$ and λ terms. In the case of the truncated substrate (**1**), the rate of electron transfer would be expected to be further reduced by the requirement for water molecules to complete the pathway. The actual rate for this process is difficult to predict, but it would have to be $\geq 10^5$ s^{-1} in order to lead to nearly identical rates for **1** and **2**. This limit may impose too large a constraint for a "through substrate" electron pathway contributing to $k_{cat}/K_m(O_2)$. On the other hand, if a rapid electron transfer follows C−H cleavage (see below), its rate would only need to be in excess of the reported values for k_{cat}, (<40 s^{-1}) to be compatible with the measured parameters. In this instance, direct electron transfer through solvent water should be fast enough to accommodate catalysis[15] and there would be no need to invoke the substrate as an electron conduit.

The aggregate data on PHM and DβM appear to be inconsistent with postulated electron-transfer mechanisms, pointing toward a mechanism in which transfer *makes use of the bulk solvent that separates Cu_H and Cu_M*. This can be visualized in the context of the mechanism proposed in Scheme 1. One key aspect of this mechanism is that the interdomain electron transfer occurs after C−H activation, reducing the distance over which electron transfer

Scheme 1. Postulated Mechanism for PHM in Which Interdomain Electron Transfer Occurs after C−H Activation[a]



[a] From ref 10.

must take place to ca. 7 Å.[10] In a related mechanism, Chen and Solomon[17] have suggested that electron transfer occurs after both C−H abstraction and oxygen insertion. While rate constants for electron transfer through water over distances greater than 10 Å are very slow,[15] a rate constant for transfer over 7 Å is expected to be significantly faster than the measured k_{cat} values of 17−37 s^{-1}. Overall, Scheme 1 and related mechanisms[17] offer a tractable resolution to the available data and to the long-standing mechanistic enigma of DβM and PAM. Given the solvent-accessible nature of the PHM site (Figure 1), investigations, for example, of the impact of mixed solvents on the enzymatic catalytic efficiency of DβM and/or PAM may prove to be instructive regarding the proposed "through water" electron-transfer process.[18]

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