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Enzymatic Methyl Transfer: Role of an Active Site Residue in Generating Active Site Compaction That Correlates with Catalytic Efficiency

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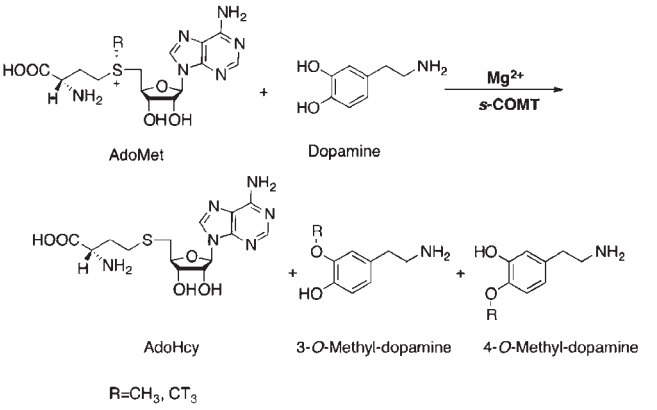
Supporting Information

ABSTRACT: Human catechol-*O*-methyltransferase (COMT) catalyzes a methyl transfer from *S*-adenosylmethionine (AdoMet) to dopamine. Site-specific mutants at three positions (Tyr68, Trp38, and Val108) have been characterized with regard to product distribution, catalytic efficiency, and secondary kinetic isotope effects. The series of mutations at Tyr68 within wild-type protein and the common polymorphic variant (Val108Met) yields a linear correlation between the catalytic efficiency and the size of the secondary kinetic isotope effect. We conclude that active site compaction in COMT is modulated by a proximal side chain residing behind the sulfur-bearing methyl group of AdoMet. These findings are discussed in the context of the active site compression that has been postulated to accompany enzyme-supported hydrogen tunneling.

The remarkable rate acceleration achieved by enzymes, ca. 10^{20} faster than the uncatalyzed reaction,[1] has fascinated researchers for almost a century. An understanding of the physical origins of this catalytic effect has wide ranging implications for the *de novo* design of new catalytic materials. While the proposal of Pauling of enhanced transition state binding as the origin of catalysis[2] held sway for many decades, the model is a static one and breaks down in the context of the extensive evidence for enzyme-supported hydrogen tunneling. The repeated demonstration of hydrogen tunneling behavior with weak or temperature-independent kinetic isotope effects (KIEs) has led to a new model in which enzymes can create active site geometries with H-donor/acceptor distances that are reduced to ca. 2.8 Å as a prerequisite for efficient hydrogen tunneling: a dynamical model has emerged in which conformational sampling within the protein allows the transient achievement of these short donor/acceptor distances.[3−5] An additional important feature is the modulation of the reactive internuclear distance by one or more bulky amino acid side chains in the enzyme active site.[6] Until now, it has been uncertain whether the requirement for a compressed active site geometry was specific to tunneling reactions or was characteristic of all enzyme reactions (cf. refs 5,7). To address this important question, we have studied the properties of an enzymatic group transfer of atomic mass 15 (methyl group) for comparison to the transfer of lighter particles with masses of 1 to 3 (protium, deuterium, and tritium).

Scheme 1



Methyl group transfer is a fundamental biological reaction encompassing processes as diverse as metabolite modification and gene regulation.[8,9] Our focus is on the reaction catalyzed by COMTs, in which a methyl group is transferred from AdoMet to a reactive oxygen nucleophile on a catechol nucleus (dopamine in Scheme 1), forming *S*-adenosyl-L-homocysteine (AdoHcy) and methylated catechol (3-methoxytyramine and 4-methoxytyramine). This process effectively removes dopamine from its signaling pathway, targeting the neurotransmitter for degradation.[10] The monomeric COMT proceeds by an ordered mechanism with the coenzyme (AdoMet) binding first, followed by a divalent cation (Mg^{2+}), and finally a catechol substrate.[11] The chemical step of methyl transfer in the COMT from rat liver had previously been demonstrated to display an inverse secondary deuterium kinetic isotope effect of 0.83 ± 0.05 in contrast to the model reaction where the KIE is close to unity or even higher.[12−14] The more inverse KIE on the enzyme was initially interpreted in the context of a compressed S_N2 transition state,[14−18] though subsequent computational work failed to accomplish shorter internuclear distances at the transition state of the enzymatic reaction.[19−21]

From the crystal structure of the human, soluble form of enzyme, *s*-COMT,[22] the side chain of an active site Tyr68 is seen to be positioned directly above the AdoMet, with a short distance (3.5−3.9 Å[23]) between the sulfur atom of AdoMet and the beta carbon of Tyr68 (Figure 1). Site-specific mutagenesis has been

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used to generate Y68F and Y68A, with the goal of correlating the magnitude of the secondary KIE to changes in catalytic efficiency. Controls have involved mutagenesis at positions in *s*-COMT either distal from the active site or residing in a different region of the active site. In the former case, we exploited the observation of a coding polymorphism in human COMT that maps to documented disease states and shows the substitution of methionine for valine at codon 108, about 20 Å from the active site,[22] Figure 1. In a second control, Trp38, which is a "gatekeeper" holding catechol in the correct orientation for methylation,[23] was mutated to Ala and Val.

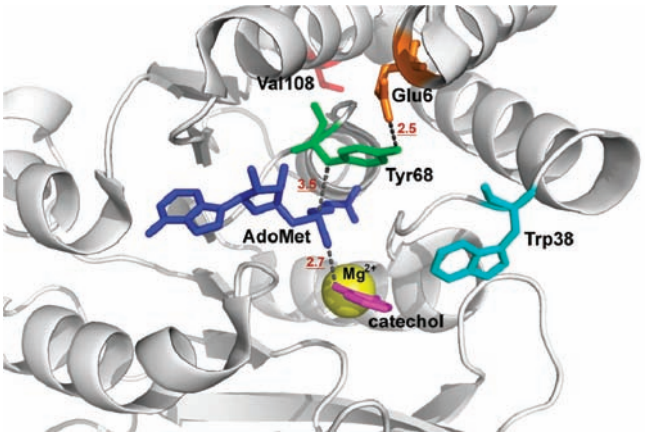

Figure 1. Active site of human WT *s*-COMT complexed with catechol (magenta), AdoMet (blue), Mg^{2+} (yellow), residues Glu6 (orange), Trp38 (cyan), Tyr68 (green), and Val108 (red) shown in stick representation (PDB: 3BWM). Distance values (in firebrick) are underlined with unit Å.

Table 1. *Meta*/*Para* Methylation Ratio of Dopamine Catalyzed by the Recombinant Human *s*-COMT and Its Mutants

s-COMT	meta/para[a]
108V(WT)	4.8 ± 0.4
Y68F	4.6 ± 0.4
Y68A	4.6 ± 0.6
V108M	5.1 ± 0.6
V108M/Y68F	4.7 ± 0.2
V108M/Y68A	4.2 ± 0.4
W38A	1.7 ± 0.1
W38V	1.9 ± 0.03

[a] The *meta*/*para* methylation ratio was calculated according to the total net counts for ^{14}C in the product.

In anticipation of relatively small differences among the 2° KIE for the wild-type *s*-COMT in relation to its mutants, we pursued a method of measurement that is capable of greater precision than the direct comparison of protio- vs deuteriomethyl group transfer. This involves an internal competition between protium and tritium at the transferred methyl group, while using a remote label to monitor the movement of the all protio-substituent. The experiments were, thus, carried out by mixing a trace of all tritio- (*S*-adenosyl-L-[methyl-^{3}H]methionine)[24] in protonated AdoMet with a ^{14}C-labeled dopamine according to procedures outlined in Supporting Information (SI). The *meta* and *para* products were isolated and quantified against authentic standards, and the $^3H/^{14}C$ ratio in each peak determined by liquid scintillation counting. Using WT *s*-COMT, the substrate is predominantly methylated at the *meta*-position[10] and this pattern is retained for the mutants at positions 68 and 108 (Table 1). The competitive k_{cat}/K_m 2°-tritium KIEs are obtained according to eq S1, with experimental values for WT *s*-COMT of 0.791 ± 0.012 and 0.775 ± 0.060, for *meta*- and *para*-methylation, respectively (Tables 2 and S1). The higher yield for the *meta*-product correlates with higher precision KIEs, leading to a primary focus on the data for formation of 3-*O*-methyl dopamine (Table 2). The comparative trends for the 4-*O*-methyl product are presented in Table S1 and Figure S1.

As the side chain at position 68 is changed from Tyr (WT) to Phe or Ala, both k_{cat} and k_{cat}/K_m are reduced, Table 2. Whereas, we have not been able to assess the rate-determining step on k_{cat}, the 1° ^{14}C-KIE for transfer of the labeled methyl group from AdoMet to the *meta* position of dopamine (1.063 ± 0.020) is consistent with a rate-determining methyl transfer under conditions of k_{cat}/K_m (see Table S6). The experimental k_{cat}/K_m values are quite sensitive to the mutagenesis, undergoing a 100−200 reduction in k_{cat}/K_m(AdoMet) and a 600−1200 reduction in k_{cat}/K_m(Dopamine) (Table 2). In contrast to position 68, the distal variant, V108M, shows only a small change in activity.

Table 2. Kinetic Parameters and Secondary Kinetic Isotope Effect (2° KIE) for the 3-*O*-Methylation of Dopamine Catalyzed by the Recombinant Human *s*-COMT and Its Mutants

s-COMT	k_{cat}(min^{-1})	k_{cat}/K_m (M^{-1} s^{-1}) Dopamine	k_{cat}/K_m (M^{-1} s^{-1}) AdoMet	2° KIE k_{CH3}/k_{CT3}[a]
108V(WT)	12.3 ± 0.3	833 ± 55	3203 ± 359	0.791 ± 0.012 (0.850)
Y68F	21.9 ± 0.9	127 ± 14	1370 ± 234	0.822 ± 0.021 (0.873)
Y68A	0.81 ± 0.05	2.6 ± 0.2	45 ± 7	0.850 ± 0.012 (0.893)
V108M	25.4 ± 0.5	1362 ± 71	3683 ± 708	0.784 ± 0.014 (0.845)
V108M/Y68F	23.1 ± 1.6	139 ± 23	1269 ± 189	0.822 ± 0.007 (0.873)
V108M/Y68A	0.50 ± 0.01	1.1 ± 0.1	20 ± 4	0.863 ± 0.019 (0.903)
W38A	4.0 ± 0.3	14 ± 2.1	567 ± 140	0.802 ± 0.023 (0.858)
W38V	2.7 ± 0.2	7.6 ± 1.3	437 ± 100	0.814 ± 0.015 (0.867)

[a] KIE reported is for the second-order rate constant, k_{cat}/K_m. The values in parentheses are k_{CH3}/k_{CD3}, calculated according to $\ln(k_{CH3}/k_{CT3})/\ln(k_{CH3}/k_{CD3}) = 1.442$.

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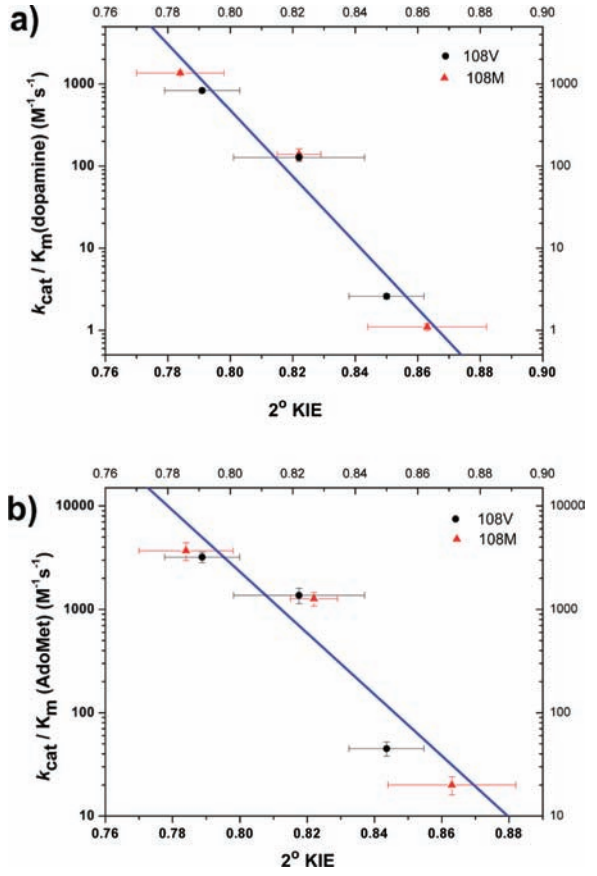

Figure 2. Relationship between the k_{cat}/K_m and 2° KIE for 3-*O*-methylation catalyzed by Y68 mutants of *s*-COMT; data are presented for both WT and the V108M variants. (a) k_{cat}/K_m(Dopamine) vs the corresponding 2° KIE (k_{CH3}/k_{CT3}); $r^2 = 0.96$. (b) k_{cat}/K_m (AdoMet) vs the corresponding 2° KIE (k_{CH3}/k_{CT3}); $r^2 = 0.90$. Data in black refer to the WT series; data in red refer to the V108M series.

The second proximal mutant, W38A, while reduced in activity in relation to WT, remains more active than Y68A (Table 2). Thus, from the point of view of activity, changes in Tyr68 have the greatest impact. This is mirrored in the size of the KIEs, which are similar for WT and V108M and their respective Y68 mutants, and within experimental error for the WT and W38 variants.

The relationship between the magnitude of the 2° KIE and the second-order rate constants for the Y68 series is plotted for the substrates dopamine and AdoMet in Figure 2a and b. There are statistically significant trends in which the 2° KIE goes from ca. 0.78 to ca. 0.86 as the rate diminishes over 3 and 2 orders of magnitude, respectively. We note that the competitive KIEs, which represent k_{cat}/K_m measurements, must arise from transition state differences since, in all cases, the ground state refers to unbound substrates.[25] Since the magnitude of these 2° KIEs is primarily determined by the looseness of the transition state, the more inverse the 2° KIE, the tighter/stiffer the enzymatic transition state.[18,26] In no instance do the mutants approach the KIE close to unity seen in solution,[12−14] indicating the retention of considerable active site compaction even in the case of Y68A. This implies that the creation of reactive configuration(s) on the enzyme occurs via a cooperative process requiring multiple protein side chains and native protein structures. While we cannot yet posit a precise theoretical basis for the linear relationships shown in Figure 2, the 1:1 correlation between the catalytic efficiency and magnitude of the secondary KIE in a methyl group transfer demonstrates a strong interdependence between active site stiffness and catalytic efficiency.

From the point of view of enzyme structure, *s*-COMT belongs to the AdoMet-dependent methyltransferase family, containing a central core with seven-stranded mixed β-sheets sandwiched between two layers of α-helices.[27] According to a Ramachandran plot,[22] the side chain of residue Tyr68 is located in a loop between strand β1 and helix α4 that falls within the disallowed region. The hydrogen bond formed between the oxygen of Glu6 and the hydroxyl group on the Tyr68 stabilizes the unfavorable conformation (Figure 1). Upon disruption of this hydrogen bond, the mutant side chain Phe is expected to be free to fluctuate, impairing the precise positioning of the transferred methyl group. For the case of Y68F, the experimental K_m for AdoMet is increased 3- to 4-fold and ca. 10-fold for dopamine (Table S2). The effects seen for Y68A are somewhat greater, with K_m increasing ca. 4- and 22-fold for AdoMet and dopamine, respectively. The fact that K_m values for both substrates are increased indicates a synergistic effect of Y68 that is propagated into the catechol-binding site. These increases in K_m, though not necessarily equivalent to K_d values, suggest moderate ground-state effects that are significantly less than the impact of mutation on k_{cat}/K_m (Figure 2 and Table 2).

In the case of Trp38, X-ray data and theoretical calculations indicate its role in generating a hydrophobic pocket for catechol binding. The perturbed protein, W38A or W38V, has a very significant impact on the K_m for catechol, with little influence on the K_m for AdoMet (Table S2). The differential impact of the Y68 and W38 mutants on the respective K_m values for AdoMet and dopamine is not unexpected, given the more remote positioning in the W38 active site. The similarity of the 2° KIEs for WT and W38 mutants argues against a direct role for W38 in altering the interaction between the methyl group donor and acceptor.

For S_N2-type reactions, computational studies have identified the C_α-H(D,T) stretching vibration as an important contributor to the magnitude of the KIE, with the out-of-plane bending vibrations playing a significant role in the trends of the KIE.[18] Early modeling of the methyl transfer reaction catalyzed by COMT led to the conclusion of a more compressed transition state for the enzymatic reaction in contrast to the model reaction.[14,15] Application of QM/MM methodologies to simulations of methyl transfer in an enzymatic vs nonenzymatic context found little difference between these two systems with regard to internuclear distances,[19−21] although the enzymatic active site was concluded to be stiffer.[20] One possible limitation of the cited simulations of the enzymatic methyl transfer coordinate is the manner in which the QM region is defined and quantified. Additionally, protein conformational sampling (on time frames that span ms to fs) has been shown to be essential for efficient hydrogen tunneling[5] and, even when incorporated into simulations of methyl transfer, may be on timescales that are too long to be detected with current methodologies. The experimental generation of active site mutants of COMT that show a 1:1 relationship between catalytic efficiency and the magnitude of 2° KIEs provides, thus, an important test of the hypothesis of compression, as well as a goal for computational chemists to provide a physically meaningful rationalization of the demonstrated impact of a neighboring amino acid side chain on the 2° KIE. One central issue requiring clarification is the origin of a transition state structure on the enzyme that can be described as stiffer than the

corresponding solution reaction but not reduced in internuclear distances. The difference in reaction coordinate distances among the Tyr68 mutants may be small, with Schowen and co-workers suggesting a reduction of 0.03−0.29 Å between the donor and acceptor atoms on the WT-enzyme relative to the uncatalyzed reaction.[14]

According to the most current models of H-tunneling, the protein catalyst must be able to sample a range of conformational substrates that differ with regard to the strength of interactions between the bound substrate and enzyme and the internuclear distance between reactants. In this scenario, only a fraction of the total enzyme will achieve, transiently, the family of substrates that create the tunneling-appropriate H-donor to acceptor distance of ca. 2.8 Å.[5] In a much earlier study of horse liver alcohol dehydrogenase, a single residue that resides behind the nicotinamide ring of the redox cofactor NAD was mutated to smaller side chains leading to a concomitant reduction in both catalytic efficiency and the degree of deviation of the Swain−Schaad relationship from its nontunneling, semiclassical limit.[28] This correlation was later interpreted in the context of a change in active site compression that was initially optimal in the WT enzyme and reduced toward that of a solution reaction in the mutants.[29] The reduction in distance that has been predicted to be a requirement for effective H-tunneling is of the order of 0.4 Å.[5] While future studies of binding isotope effects will be necessary to eliminate unusual ground state effects in enzymatic methyl transfer, the overall similarity between the role for proximal bulky side chains in enzymatic H-transfer and the present findings point toward a general picture of enzyme catalysis in which protein conformational sampling can create transient active site configurations that are stiffer and distinct from the activated complexes in solution. A key element, in need of future exploration, is the precise manner by which dynamical excursions within the protein backbone can be transmitted to proximal side chains positioned to generate reaction coordinate compaction. Such an understanding appears fundamental to our ability to design new catalysts with turnover rates approaching naturally occurring enzymes.

■ ASSOCIATED CONTENT

Supporting Information. Materials and methods, detailed description of the competitive KIE measurements, Tables S1−S6, Figures S1−S2, and supporting references. This material is available free of charge via the Internet at http://pubs.acs.org.

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