A Chemical Model of Catechol-*O*-methyltransferase. Methylation of 3,4-Dihydroxybenzaldehyde in Aqueous Solution[1)]

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The reaction of 3,4-dihydroxybenzaldehyde (LH_2) and dimethylsulfate (DMS) to form the *m*- and *p*-*O*-methylated products (vanillin and isovanillin, respectively) in aqueous 2-(*N*-morpholino)ethane sulfonate buffer was studied kinetically. The products were determined by means of high-performance liquid chromatography. The *O*-methylation occurred principally at the *p*-hydroxyl group in the absence of divalent metal ions. In the presence of Cu(II), the *m*-methylation was promoted and became predominant. Zn(II) showed a similar but less pronounced effect. The effects were explained in terms of the complex formation of LH_2. The second order rate constants for the *m*- and *p*-methylation of the species, LH_2, CuL and CuL_2^{2-} by DMS were calculated. The values and their ratio for the *m*-/*p*-reactions increased in the order of $LH_2 < CuL < CuL_2^{2-}$. The reaction may serve as a chemical model for catechol-*O*-methyltransferase, which requires divalent metals and catalyzes the *m*-methylation.

Keywords 3,4-dihydroxybenzaldehyde; dimethylsulfate; vanillin; isovanillin; kinetics; *O*-methylation; metal chelate; copper-(II); catechol-*O*-methyltransferase; enzyme model

Catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) catalyzes the transfer of the methyl group of *S*-adenosyl-L-methionine (SAM) to the phenolic group of catechol or substituted catechols. The enzyme is present in the soluble and microsomal fractions of cells and tissues and plays an important role in the metabolism of catecholamines. It requires divalent metal ions such as Mg^{2+} for the catalysis. The *O*-methylation *in vivo* occurs almost exclusively on the *m*-hydroxyl group of the catechols, but *in vitro* it occurs at one of the *m*- or *p*-hydroxyl groups.[2)]

The role of metal ions in nonenzymatic and enzymatic *O*-methylation of catechol derivatives was studied by Senoh and coworkers.[3)] They examined the catalytic effects of various metal ions in both nonenzymatic and enzymatic reactions and proposed a reaction mechanism involving the metal complex of catechol. However the results of their kinetic studies were limited due to the lack of a facile analytical method for the methylated products.

Recent developments in high-performance liquid chromatography (HPLC) have provided facile and accurate methods of analysis for catechol derivatives.[4)] We therefore analyzed the products of nonenzymatic *O*-methylation of 3,4-dihydroxybenzaldehyde by dimethylsulfate by means of HPLC and studied the kinetics of the reaction in the presence and absence of metal ions, as a possible chemical model of COMT. The reaction is formulated as shown in Chart 1.

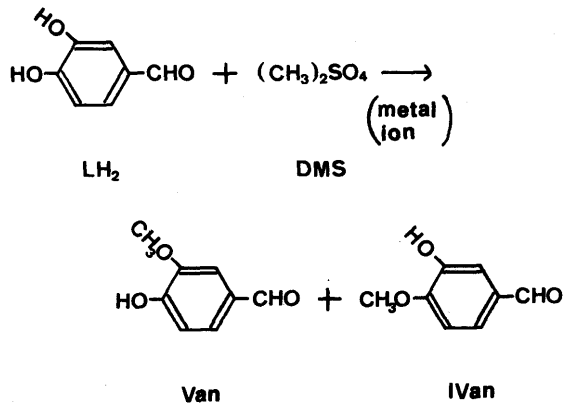

Chart 1

Experimental

Materials Chemicals were obtained from commercial sources. 3,4-Dihydroxybenzaldehyde (LH_2) was recrystallized from toluene, and vanillin and isovanillin from water, prior to use. Deionized and distilled water was used. Other chemicals were of reagent grade.

Kinetic Runs Kinetic runs were carried out in 0.2 M MES buffer (2-(*N*-morpholino)ethane sulfonic acid–NaOH, pH 5.6) solution. A solution of LH_2 in the buffer was preincubated at a fixed temperature with or without a divalent metal ion. Dimethylsulfate (DMS) in 55 μl of ethanol was added and the mixture (5 ml) was placed in a thermostated bath with shaking. A portion of the mixture was withdrawn at predetermined intervals, chilled in ice and acidified (pH < 2) with 6 M HCl. A 100-μl volume of 2 mM ethylvanillin in ethanol was added. The *O*-methylated compounds were extracted with 5 ml of water-saturated chloroform by shaking vigorously for 5 min. The chloroform extract (4 ml) was washed with water and concentrated *in vacuo* and the residue was dissolved in ethanol. A portion of the ethanol solution was injected into the HPLC.

HPLC Measurements A Hitachi model 655 liquid chromatograph equipped with a UV monitor operated at 272 nm and a model 655-61 data processor was used. The column was Zorbax Sil (Shimadzu, 250 × 4.6 mm i.d.). The mobile phase was *n*-hexane–ethanol (9 : 1, v/v) and its flow rate was 1 ml/min at a pressure of about 70 kg/cm^2. The retention times of ethylvanillin, vanillin and isovanillin were 7.7, 9.8, and 12.0 min, respectively. Vanillin and isovanillin in the reaction mixture were determined from the ratio of their peak areas to that of ethylvanillin, the internal standard. The retention time of LH_2 was 10.5 min, though part of the unreacted LH_2 was washed down to the aqueous layer.

pH Measurement Values of pH of aqueous DMS were measured with a Horiba model F-8 AT pH meter in a thermostated vessel with thorough stirring.

Results and Discussion

Kinetic Runs Figure 1 shows a time course of the formation of vanillin (Van, *m*-methylation) and isovanillin (IVan, *p*-methylation) from LH_2 and DMS in the absence of divalent metal ions. The formations of Van and IVan followed first-order kinetics and their rates (V) were expressed as

$$V = dS/dt = k_{obs}(S_\infty - S)$$

where k_{obs} indicates the observed first-order rate constant and S_∞ and S are the concentration of Van or IVan at the completion of the reaction and at t, respectively. In-

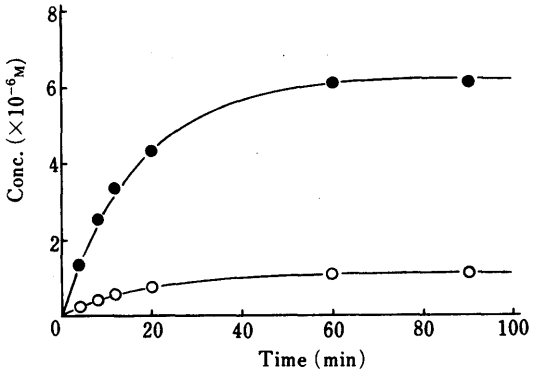

Fig. 1. Time Dependence of *O*-Methylation of 3,4-Dihydroxybenzaldehyde at 37 °C in the Absence of Divalent Metal Ion

Open circles, vanillin; closed circles, isovanillin. $[LH_2]_0=[DMS]_0=10$ mM.

tegration of the equation gives

$$\ln[S_\infty/(S_\infty-S)]=k_{obs}t \quad (1)$$

Also we can obtain $V_0=k_{obs}S_\infty$, where V_0 indicates the initial rate of the reaction, defined as V at $t=0$. The observed constants, k_{obs}, were almost the same for Van and IVan, though S_∞ of IVan was several times larger than that of Van under the conditions used.

The dependences of k_{obs} and S_∞ on the initial concentrations of LH_2 ($[LH_2]_0$) and DMS ($[DMS]_0$) were examined in the ranges of 10 to 40 mM. The rate constant, k_{obs}, did not vary with the initial concentrations, whereas the values of S_∞ and hence those of V_0 were proportional to $[LH_2]_0$ and $[DMS]_0$. The total yield of Van and IVan at the completion of the reaction was less than 1% of the amount expected from those of LH_2 and DMS.

The first-order kinetics were followed in all runs throughout the present study. The constants k_{obs} were practically unaffected by the presence of metal ions. On the other hand, the value of V_0 as well as the ratio of *m*- to *p*-methylation was dependent on the kind and the concentration of divalent metal ions. V_0 was proportional to $[DMS]_0$, but not to the total concentration of M^{2+}, $[M^{2+}]_T$. The variations of V_0 against the total concentrations of Cu(II) and Zn(II) are shown in Figs. 2 and 3, respectively. The presence of Cu(II) enhanced the *O*-methylation of the catechol, especially that at the *m*-position. Zn(II) showed a similar but less pronounced effect. No significant enhancement was observed in the case of Mg(II) (data not presented).

The temperature dependence of k_{obs} was measured. The values of k_{obs} were $7.54\times10^{-4}\,s^{-1}$ (30 °C), $1.01\times10^{-3}\,s^{-1}$ (37 °C), $1.81\times10^{-3}\,s^{-1}$ (44 °C) and $3.45\times10^{-3}\,s^{-1}$ (51 °C). The Arrhenius plot of the values was almost linear.

Hydrolysis of DMS The constancy of k_{obs} was explained in terms of rapid hydrolysis of DMS in the solution, as described in the following section. The hydrolysis of DMS to monomethylsulfate was reported to be a rather rapid first-order process,[5] whereas that of monomethylsulfate to sulfuric acid is a slower step.[6] These reports suggest that the principal methylating species in the reaction is DMS.

The kinetics of the hydrolysis of DMS were measured in 10 mM aqueous unbuffered solution. The hydrogen ion concentration of the solution increased in a first-order manner. The first-order rate constant, k_d, was $5.85\times10^{-4}\,s^{-1}$ at 37 °C. The value for 37 °C calculated from the data reported by Robertson and his coworker[5] was $6.71\times10^{-4}\,s^{-1}$. These values are close to each other and also close to the values of k_{obs} obtained in the present study.

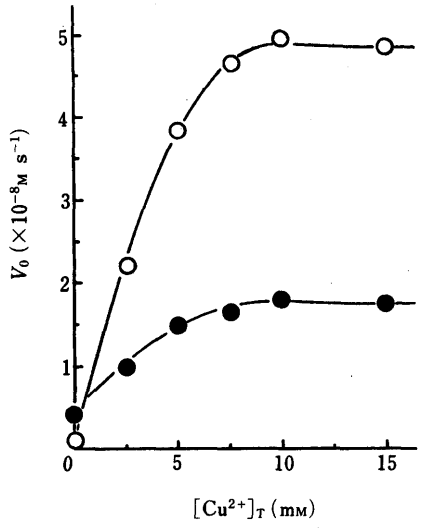

Fig. 2. Cu(II)-Catalyzed *O*-Methylation of 3,4-Dihydroxybenzaldehyde at 37 °C

Open circles, vanillin; closed circles, isovanillin. $[LH_2]_0=[DMS]_0=10$ mM.

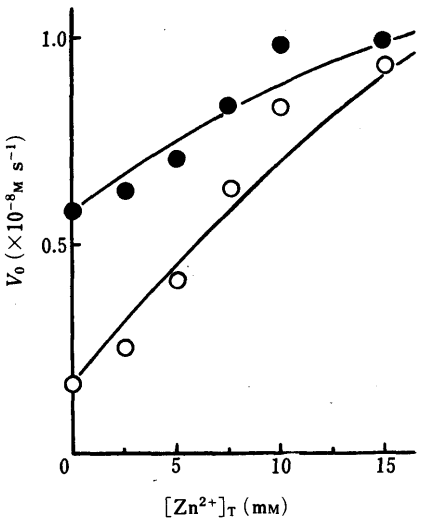

Fig. 3. Zn(II)-Catalyzed *O*-Methylation of 3,4-Dihydroxybenzaldehyde at 37 °C

Open circles, vanillin; closed circles, isovanillin. $[LH_2]_0=[DMS]_0=10$ mM.

Kinetic Analysis The fact that V_0 was proportional to the initial concentrations of the reactants, $[LH_2]_0$ and $[DMS]_0$, suggests that the reaction is first order with respect to both $[LH_2]$ and [DMS]. Then V in the absence of the metal ion can be expressed as

$$V=k_1[LH_2][DMS] \quad (2)$$

where k_1 indicates the second-order rate constant.

Since the yields of the products were small, we can replace $[LH_2]$ by $[LH_2]_0$. DMS was rapidly hydrolyzed in the medium with the rate constant k_d and only a negligible amount reacted with LH_2. Thus its concentration was

assumed to be $[DMS]_0 \exp(-k_d t)$ and V can be expressed as

$$V = dS/dt = k_1[LH_2]_0[DMS]_0 \exp(-k_d t) \quad (3)$$

The initial rate of the reaction, V_0, should be equal to $k_1[LH_2]_0[DMS]_0$. Then we obtain

$$dS/dt = V_0 \exp(-k_d t)$$

Integration of the equation gives

$$S = (V_0/k_d)[1 - \exp(-k_d t)]$$

Since S approaches S_∞ with the progress of the reaction, the equation can be written as

$$S = S_\infty[1 - \exp(-k_d t)]$$

which can be transformed to

$$\ln[S_\infty/(S_\infty - S)] = k_d t \quad (4)$$

We can conclude $k_d = k_{obs}$ from the comparison of Eqs. 1 and 4. This conclusion can well explain the experimental results. The second-order rate constants, k_1, for the formations of Van and IVan were calculated and are included in Table I.

Kinetics of the Metal Ion Catalysis In the presence of divalent metal ions (M^{2+}), both the uncomplexed form of the reactant, LH_2, and the complexes, ML and ML_2^{2-}, should react with DMS. For obtaining the concentrations of these species, the stability constants of the complexes must be known.

The conditional stability constants of the complexes of LH_2 with some divalent metal ions (M^{2+}), defined as $K_1 = [ML]/[M^{2+}][LH_2]$ and $K_2 = [ML_2^{2-}]/[ML][LH_2]$, were measured in 0.2 M MES buffer (pH 5.6) at various temperatures from the variation of the absorption at around 350 nm. Details of the measurements and the results of the Cu(II) and Zn(II) complexes were reported elsewhere.[7] $[LH_2]$, [ML] and $[ML_2^{2-}]$ were calculated from the values of K_1 and K_2 and the total concentrations of LH_2 and M^{2+}.

In the metal ion-catalyzed reactions, V_0 can be expressed as

$$V_0 = (k_1[LH_2] + k_2[ML] + k_3[ML_2^{2-}])[DMS]_0 \quad (5)$$

The second-order rate constants, k_2 and k_3, were obtained from the kinetic data. The results are shown in Table I. As K_2 for Zn(II) was too small to obtain a reliable value, k_3 was not calculated. The curves in Figs. 2 and 3 are drawn from the values of the rate constants and Eq. 5. The good fit with the observed data supports the proposed mechanism.

Reactivity of the Metal Complexes The presence of Cu(II) enhanced the methylation at the *m*-OH much more than that at the *p*-OH of the catechol. Zn(II) showed a similar but less distinct effect. No significant effect was observed in the case of Mg(II). The order of the catalytic activities seems to be related to the complex stability constants.[1a] The stability constant of the Zn(II) complex was much smaller than that of Cu(II) and the Mg(II) complex formation was negligible under the conditions used.

The reactivity of the uncomplexed and complexed species of the catechol can be estimated from the second-order rate constants. The magnitude of the constants in the Cu(II) catalysis was in the order of $k_1 < k_2 < k_3/2$. Since k_3 is the constant for the 1:2 complex, ML_2^{2-}, $k_3/2$ was compared with k_1 and k_2.

The catalytic activities of Cu(II) for the *m*- and *p*-methylation can be estimated from the ratio of the constants of the two reactions. In the uncomplexed species the ratio of k_1 of the *m*- to that of the *p*-methylation was less than unity. The ratios of k_2 and k_3 were 2.78 and 3.44, respectively. These values show that the *p*-methylation was the favored pathway in the reaction of the uncomplexed catechol, while the *m*-methylation to form Van was favored in the 1:1 complex, ML, and much more favored in the 1:2 complex, ML_2^{2-}.

Activation Parameters Estimation from the complex stability constants indicated that 99.2% to 99.4% of the reactant is present as the 1:1 Cu(II) complex, CuL, at $[LH_2]_T = 10$ mM and $[Cu(II)]_T = 15$ mM at 30—51 °C. Under these conditions, the reaction can be regarded as that between CuL and DMS. From the temperature dependence of the rate of the Cu^{2+}-catalyzed ($[Cu]_T = 15$ mM) and uncatalyzed reactions, the activation parameters at 37 °C were calculated for k_1 and k_2 of Van and IVan formation, and the values obtained are listed in Table II.

The increases in the k values in the 1:1 Cu(II) complex are the consequence of the decreases in $\Delta G^{\ddagger}$. The activation parameters obtained indicate that it is the increase in $\Delta S^{\ddagger}$ and not the decrease in E_a or $\Delta H^{\ddagger}$, which contributes to the decrease in $\Delta G^{\ddagger}$. The increase in $\Delta S^{\ddagger}$ was more prominent in the *m*- than in the *p*-methylation.

Implications for Enzymatic Catalysis The *O*-methylation *in vivo* by the enzyme occurs almost exclusively on the *m*-hydroxyl group of the catechols. On the other hand, the nonenzymatic *O*-methylation by DMS occurs principally at the *p*-hydroxyl group of the uncomplexed catechol. The *m*-methylation was promoted and became predominant in the Cu(II)-catalyzed nonenzymatic reaction. The effect of Cu(II) was explained in terms of increased reactivity of the complexes. Thus the Cu(II) catalysis may serve as a good model for the enzyme, COMT.

The mechanism of the enzymatic and nonenzymatic *O*-methylation may be regarded as a displacement reaction on

TABLE I. The Second-Order Rate Constants of *O*-Methylation

Metal	Product	Second-order rate constant ($M^{-1}s^{-1}$) k_1	k_2	k_3
None	Van	1.41×10^{-5}		
	IVan	5.85×10^{-5}		
Cu(II)	Van		4.86×10^{-4}	2.07×10^{-2}
	IVan		1.75×10^{-4}	6.02×10^{-3}
Zn(II)	Van		2.70×10^{-4}	—
	IVan		1.95×10^{-4}	—

TABLE II. Activation Parameters

Parameter	Cu^{2+}-uncatalyzed		Cu^{2+}-catalyzed[a]	
Product	Van	IVan	Van	IVan
$\Delta G^{\ddagger}$ (kJ mol^{-1})	105.8	100.6	95.9	98.2
E_a (kJ mol^{-1})	55.0	78.2	71.7	86.9
$\Delta H^{\ddagger}$ (kJ mol^{-1})	52.4	75.6	69.1	84.4
$\Delta S^{\ddagger}$ (JK^{-1} mol^{-1})	−172.2	−80.5	−86.4	−44.6

a) $[Cu^{2+}]_T = 15$ mM.

the methylating agent by nucleophiles such as phenolic or phenolate groups of the reactant and water. In the two phenolic groups of LH_2, the *p*-OH is supposed to be more acidic and more readily methylated than the *m*-OH. The *m*- and *p*-phenolate should form the metal chelate in the divalent metal complexes. The metal chelation may increase the nucleophilicity of the *m*-phenolate. Further increase in the nucleophilicity is expected in the 1 : 2 chelate, ML_2^{2-}, an anionic species. Thus the *m*-methylation would be favored in the Cu(II) complexes.

COMT was reported to require Mg(II) or other divalent metals for activity.[2,3] The enzymatic reaction may proceed through a species in which one of the ligands (L) in ML_2^{2-} of the present model is replaced by a chelating group of the enzyme.

Mg(II) did not show significant catalysis in the present nonenzymatic reactions. This can be ascribed to the fact that the Mg(II) chelate of LH_2 was hardly present in aqueous media. The enzymatic reaction should proceed in more hydrophobic environments where effective chelation of Mg(II) occurs. We recently found[8] that in methanol, where the Mg(II) chelate formation is more favored than in water, Mg(II) showed a considerable catalytic activity in a nonenzymatic *O*-methylation of LH_2. Details of the study will be reported shortly.

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