Spectrophotometric Kinetic Studies and Substrate Inhibition of Hog Kidney Histaminase Activity by an Improved 2,4-Dinitrophenylhydrazine Method

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Abstract—We improved the experimental procedure for the measurement of hog kidney histaminase activity using histamine as a substrate on the basis of a spectrophotometric estimation of the 2,4-dinitrophenylhydrazone of imidazole acetaldehyde and studied the steady-state kinetics to obtain the basic data for further investigations of the oxidative deamination of histamine. The initial and mean velocities of the enzymatic reaction were calculated and plotted against the amount of enzyme. It was found that the initial velocity increased linearly. The time t_α necessary to reach the extent of reaction α was calculated and plotted against the reciprocal of the enzyme concentration e_0 . It was found that t_α was linearly proportional to $1/e_0$. From Lineweaver-Burk plots, inhibition by high concentration of substrate was evident, and the v - pS curve was bell-shaped, with a pS maximum at 3.2. K_m and V were obtained: $K_m = 7.7 \times 10^{-5}$ M, $V = 0.0026$ $\mu\text{mol}/\text{min}$ (0.00075 $\mu\text{mol}/\text{min}/\text{mg}$ protein). It was concluded that our DNP method was useful for the measurement of hog kidney histaminase activity using histamine as a substrate, basic steady-state kinetic studies and further investigations of substrate inhibition and inhibitory effect.

Hog kidney diamine oxidase (DAO), or histaminase (EC 1.4.3.6), catalyzes the oxidative deamination of primary diamines and histamine and weakly catalyzes the reaction of some monoamines.

Several spectrophotometric assays have been developed for the estimation of hog kidney DAO activity (1–5). Tourkov et al. (1) reported the *p*-nitrophenylhydrazine method with *p*-dimethylaminomethylbenzylamine as a substrate. This was based on spectrophotometric measurement of the amount of *p*-nitrophenylhydrazone of the aldehyde, a product of the enzymatic reaction, in strongly alkaline medium. They also investigated, in detail, optimal experimental conditions for quanti-

tative estimation of the aldehyde. They demonstrated that their method was 5 times as sensitive as that based on a simple absorbance measurement at 250 nm of *p*-dimethylaminobenzaldehyde itself (2) and 25 times as sensitive as the conventional estimation of DAO activity with putrescine as a substrate (3).

Watanabe and Kamijo (4) developed the 2,4-dinitrophenylhydrazine (DNP) method, which is somewhat similar to that of Tourkov et al., and reported that the DNP method was useful for estimating hog kidney histaminase activity in the sense that this method involved estimation of the hydrazone derivative ($\lambda_{\text{max}} = 360$ nm) instead of imidazole acetaldehyde itself, with a λ_{max} in the UV region and a low molar extinction coefficient.

We reexamined and modified the method reported by Watanabe and Kamijo (4) by changing the reaction temperature from 60°C

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to 40°C and by increasing the reaction time range of 0–10 min to 0–60 min, which provided greater safety from inactivation of the enzyme, greater sensitivity and more convenience (6).

Recently, we further modified the experimental procedure in the enzymatic reaction to improve the time course curve of the reaction, to study the kinetics in more detail, and make better measurements of the inhibitory effect. The experimental results of steady-state kinetic studies and substrate inhibition based on the time course measurements by our established DNP method are reported and discussed.

Materials and Methods

Reagents: All reagents used for enzyme activity measurements were extra pure commercial products and used without further purification, as previously reported (6). The 0.5 mM DNP-ethanol solution (adjusted to pH 1.5 by 40% H₃PO₄) was freshly prepared each week.

Enzyme preparation and protein determination: The partially purified DAO solution of hog kidney was prepared as previously reported (6). Protein was estimated spectrophotometrically by the conventional UV nomogram method using the absorbances at 280 and 260 nm as shown in our previous paper (6): protein concentration, 17.4 mg/

ml; average specific activity, 0.0006 μ mol/min/mg protein at 40°C (pH 7.1).

Standard procedure of histaminase activity measurement: The scheme of the experimental procedure is shown in Fig. 1. We incubated 2.4 ml of the reaction mixture containing the enzyme and substrate in 0.1 M Na phosphate buffer (pH 7.1) or in solution without substrate as a control sample at 40°C. The test tubes containing the experimental and control samples were placed in an ice bath at intervals of 7.5 or 15 min, respectively, for a total time period of 0 to 60 min. To stop the reaction, 1.6 ml of the 0.5 mM DNP reagent (pH 1.5) was added. After shaking the reaction mixture with DNP at 60°C for 30 min, 2,4-dinitrophenylhydrazone of imidazole acetaldehyde (hydrazone derivative of the enzymatic reaction product) extracted in H₂O was estimated by measuring the absorbance at 360 nm as previously reported (4, 6).

The present procedure differed in three ways from that previously reported (6): 1) The volume used was 1/5 of that previously used. The volume of the reaction mixture was 2.4 ml in a 15×150 mm test tube. 2) The reaction mixture was incubated without shaking, since shaking to increase oxygenation was found to afford no advantage to the reaction rate. On the contrary, the reaction rate decreased for incubation times exceeding 30 min. The reaction test tube was opened to

Oxidative deamination of histamine by DAO(histaminase) and spectrophotometric assay by DNP method

Fig. 1. Schema of hog kidney histaminase activity measurement by the 2,4-dinitrophenylhydrazine (DNP) method.

the air, because the reaction rate was found to decrease for high concentrations of enzyme, if the reaction mixture with a stopper was incubated without shaking for various concentrations of enzyme with constant concentration of substrate. 3) To diminish the absorbance at 360 nm due to background, solvent extraction was performed two times: first by 4 ml of isooctane and then by 4 ml of CHCl_3 instead of once by 8 ml of isooctane- CHCl_3 (1:1) mixed solvent, as previously described (4, 6).

Time course measurement: The time course of the enzymatic reaction was measured every 7.5 or 15 min for a total time of 0 to 60 min for 0.1, 0.2 and 0.3 ml enzyme preparations with 3×10^{-4} M substrate concentration, according to the procedure described in the previous section. The time course measurements were also performed with substrate concentrations of 4×10^{-5} , 6×10^{-5} , 10^{-4} , 4×10^{-3} , 3×10^{-4} and 10^{-2} M using 0.2 ml of enzyme preparation.

After 2.16 ml of solution containing enzyme without substrate was preincubated at 40°C for 10 min, the test tube containing the solution was transferred to an ice bath and 1.6 ml of DNP reagent was added. After all sampling for time course measurements was terminated, 0.24 ml of 3×10^{-3} M substrate solution was added to the solution. This was used for time 0 data. The estimated absorbance values at 360 nm for the control samples (samples without substrate) were used for correction of background.

Time course measurement in the presence of inhibitor: Incubation of 2.4 ml of reaction mixture containing 0.2 ml enzyme preparation, 3×10^{-4} M substrate and an appropriate concentration of inhibitor in 0.1 M Na phosphate buffer (pH 7.1) and a control solution without substrate were carried out for various times from 0 to 60 min. The incubated solution was estimated by the DNP method using the procedure described in the standard procedure section. The estimated value for the control sample was used for correction of the background.

Calculation of mean velocity and extent of reaction: "Mean velocity" of the enzymatic reaction (product (μM)/t (min)) was calculated from the experimental values on the

time course curve. The equation used was: product (μM)/t (min) = (O.D. at 360 nm at time t)/($\epsilon \cdot t$), where ϵ is molar extinction coefficient of 2,4-dinitrophenylhydrazone of imidazole acetaldehyde (reaction product) at 360 nm in H_2O -EtOH ($\epsilon = 3.15 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) (4)).

The mean velocities for t=15, 30, 45 and 60 min were calculated, respectively, by using the experimental values on the time course curves for 0.1, 0.2 and 0.3 ml of enzyme, and these were plotted against the enzyme amount from 0 to 0.3 ml.

The time t_α minutes necessary to reach the extent of reaction $\alpha\%$ with a constant concentration of substrate (3×10^{-4} M) was calculated for 0.1, 0.2 and 0.3 ml of enzyme and plotted against the reciprocal of enzyme concentration e_0 mg/ml.

Steady-state kinetic plots: The initial velocity for the enzymatic reaction, $v = \lim_{t \rightarrow 0} [\text{product } (\mu\text{M})/t \text{ (min)}]$, was obtained from the time course curve for each of various concentrations of substrate. The initial velocities so obtained were used for the kinetic plots in this study.

Results

Improvement of time course curve and linearity check between initial velocity and amount of enzyme: Figure 2 shows the time courses for various amounts of enzyme with a constant concentration of substrate. The time course curves were apparently improved in comparison with those in our previous work (6) in which the time course for the higher concentration of enzyme began to curve early when the concentration of the product was insufficient. In the case of 0.3 ml enzyme, the absorbance at 360 nm linearly increased from 0 to 52.5 min until the value reached near 1.10. The time courses for 0.1 and 0.2 ml of enzyme were found to be linear from 0 to 60 min.

In general, the mean velocity of an enzymatic reaction is not proportional to the amount of enzyme. From a theoretical point of view, there is proportionality between the velocity and the amount of enzyme only for the initial velocity. Before plotting the initial velocity, the mean velocities for t=15, 30

Fig. 2. Time courses of various concentrations of DAO. Histamine, 3×10^{-4} M; reaction mixture, 2.4 ml in 0.1 M Na phosphate buffer (pH 7.1); incubation at 40°C .

Fig. 3. Mean velocities of histamine oxidation by DAO calculated according to the results in Fig. 2.

and 45 min were found to be proportional to the amount of enzyme for the range of enzyme from 0 to 0.3 ml. The straight line in Fig. 3

Fig. 4. Reciprocal relationship between time t_α (min) necessary to reach some extent of reaction α and concentration of enzyme e_0 (mg/ml).

coincides with the plot of initial velocity against the amount of enzyme.

The experimental results showed that in the enzyme concentration range of 0 to 0.3 ml, our experimental procedure was adequate for the activity measurement and our enzyme preparation contained no substance that suppressed or enhanced the enzymatic reaction.

Extent of reaction under constant substrate concentration: Figure 4 shows the time t_α necessary to reach some extent of reaction α plotted against the reciprocal of the enzyme concentration e_0 . The relationships between t_α and $1/e_0$ were linear.

In the integrated form of the velocity equation given by the Henri-Michaelis-Menten mechanism, $Vt = K_m \ln[s/(s-p)] + p$, reaction time t and enzyme concentration e_0 appear as a product $Vt = k_0 e_0 t$, where V is the maximum velocity; s , the initial substrate concentration; p , the concentration of product; $p = \alpha s$; and k_0 is the molecular activity. This means that under the constant initial substrate concentration s , t_α is reciprocally proportional to e_0 : $Vt_\alpha = k_0 e_0 t_\alpha = K_m \ln[1/(1+\alpha)] +$

αs . As mentioned above, our experimental results satisfied this relation between t_α and e_0 .

Kinetic parameters: The results of the time course measurements for various concentrations of substrate under 0.2 ml of enzyme are shown in Fig. 5, a and b.

The enzyme activity decreased for substrate concentrations of 3×10^{-3} M and 10^{-2} M, as compared to 3×10^{-4} M. The nature of the activity decrease was not similar to that of the activity decrease due to dilution of substrate concentration, as for instance, substrate concentration smaller than 3×10^{-4} M, as shown in Fig. 5a, where the time course curve was found to curve appreciably for the diluted concentration of substrate. The nature

Fig. 5. Time course for various concentrations of histamine. a: histamine concentrations from 4×10^{-5} to 3×10^{-4} M, b: histamine concentrations from 3×10^{-4} to 10^{-2} M. Reaction mixture, 2.4 ml in Na phosphate buffer (pH 7.1); incubation at 40°C ; DAO, 0.2 ml; s , concentration of histamine.

of the activity decrease in the high concentration region of substrate was similar to inhibition by semicarbazide ($\text{NH}_2\text{NHCONH}_2$), as shown in Fig. 6.

Figure 7 shows Lineweaver-Burk plots ($1/v-1/s$ plots) of histamine oxidation by DAO. The two points for the substrate concentrations of 3×10^{-3} M and 10^{-2} M deviated from the straight line in the substrate concentration range where there was no substrate inhibition; the Michaelis constant K_m and maximum velocity V were 7.7×10^{-5} M and $0.0026 \mu\text{mol/min}$ ($0.00075 \mu\text{mol/min/mg}$

protein), respectively.

We examined $s/v-s$ and $v-v/s$ plots for comparison and found that: $K_m = 8.8 \times 10^{-5}$ M and $V = 0.0025 \mu\text{mol/min}$ ($0.00073 \mu\text{mol/min/mg}$ protein) for $s/v-s$ plots and $K_m = 8.9 \times 10^{-5}$ M and $V = 0.0026 \mu\text{mol/min}$ ($0.00075 \mu\text{mol/min/mg}$ protein) for $v-v/s$ plots.

From the simplest velocity equation of substrate inhibition (7), where the enzyme-bisubstrate complex causing inhibition is proposed, the value of K_s' , dissociation constant of the enzyme-bisubstrate complex, which appears in the equation: $1/v = 1/V +$

Fig. 6. Time courses of histamine oxidation by DAO with and without semicarbazide ($\text{NH}_2\text{NHCONH}_2$). Histamine, 3×10^{-4} M; DAO, 0.2 ml; reaction mixture, 2.4 ml in Na phosphate buffer (pH 7.1); incubation at 40°C . [I]: concentration of inhibitor.

Fig. 7. Lineweaver-burk plots of histamine oxidation by DAO. Initial velocities ($\mu\text{M/min}$) obtained on the basis of the results in Fig. 5.

Fig. 8. Plots of initial velocity against negative logarithm of substrate concentrations. Initial velocities obtained from results in Fig. 5.

$(K_m/V) \cdot 1/s + 1/K_s'$, was roughly estimated from $1/v$ - s plots for higher concentrations of substrate to be $K_s' = 1.99 \times 10^{-2}$ M.

Figure 8 shows plots of initial velocity against the negative logarithm of substrate concentration. The obtained v - pS curve was a bell type with a pS maximum at 3.2.

Discussion

Improvement of histaminase activity measurement by the DNP method: Among the three modifications described in the section describing the standard procedure, the second one (incubation without shaking, without a stopper on the 15×50 mm test tube for the 2.4-ml reaction mixture instead of incubation with shaking, with a stopper on the 50 ml Erlenmyer flask for the 12-ml reaction mixture (6)) may be essential for the improvement of the time course curve. However, how shaking and/or the use of a stopper is related to the reaction rate in such an oxidation reaction is not clear at this time.

It is important to obtain a good time course curve for an accurate determination of initial velocity, because use of the initial velocity instead of mean velocity is essential in kinetic studies, especially in the case of a crude enzyme preparation like the one in this study. In this sense, the improvement was useful for our steady-state kinetic study.

As already mentioned (4, 6), the DNP method is useful for estimating hog kidney histaminase activity using histamine as a substrate because this method involves estimation of the hydrazone derivative ($\lambda_{\max} = 360$ nm) instead of imidazole acetaldehyde itself, which has a $\lambda_{\max}$ in the UV region, where absorptions of aromatic amino acids, proteins and other compounds are located, and has a low molar extinction coefficient, unlike the typical aromatic aldehyde. In addition to the above disadvantages of measurements in the UV region, the possible coexistence of aldehyde oxidase in the crude enzyme preparation might alter imidazole-acetaldehyde to imidazole acetic acid. Therefore, if our interest is to determine the kinetics with histamine as a substrate and/or we need accurate data of steady-state kinetics, our DNP method is more useful and more sensitive than a simple spectrophotometric measurement of the increase in the absorbance at 250 nm due to *p*-dimethylaminomethylbenzaldehyde using *p*-dimethylaminomethylbenzylamine as a substrate (2).

However, the experimental procedure in our DNP method is rather time-consuming. In this sense, the DNP method might not be very convenient for activity measurements during the course of enzyme purification. In such cases, a simple spectrophotometric assay using *p*-dimethylaminomethylbenzylamine as the substrate (2) might be more convenient than the DNP method.

Compound extracted in isooctane layer and hydrazone formation with DNP, except 2,4-dinitrophenylhydrazone of imidazole acetaldehyde: As shown by Watanabe and Kamiyo (4), the absorption spectrum of the isooctane layer has a $\lambda_{\max}$ at 335 nm, and the intensity of this band increased as the amount of enzyme increased. They described this compound as an "isooctane extractable hydrazone". Therefore, we considered that the compound extracted in the isooctane layer (see Fig. 1) was a complex of the prosthetic group containing a carbonyl group, for which van der Meer et al. and Lebenstein-Verbeek et al. (8, 9) proposed pyrroloquinolinequinone (PQQ) and DNP, or a hydrazone formed by the reaction of DNP with a trace of carbonyl compound in the partially purified enzyme.

We confirmed using small test samples of the incubated solutions (with enzyme and substrate, with enzyme but not substrate, with substrate but not enzyme, with neither enzyme nor substrate) that the isooctane layer extracted from the incubated solutions containing no enzyme gave the same spectra as those of the isooctane layer extracted from the systems containing enzyme, and the same spectrum was obtained in a simple mixture of DNP and Na phosphate buffer which was not incubated (Y. Omura et al., unpublished). Intensity increase in the isooctane layer and decrease in the CHCl_3 layer (decrease of unreacted DNP) for the incubated solution without substrate were observed as the amount of enzyme increased (10). Therefore, it is suggested that the substances causing the absorption at 335 nm in the isooctane solution were traces of unreacted DNP in the isooctane layer and the hydrazone derivative of a carbonyl compound in the crude enzyme preparation. In other words, the absorption maxima of these substances are similar to each other.

Finally, it should be noted here again that the unreacted DNP and the hydrazone derivative mentioned above were for the most part extracted in the CHCl_3 and isooctane layers, respectively, and that the traces that remained in the H_2O layer caused the background in the H_2O layer of our interest (4, 6). In our experimental procedure, the background of the absorbance at 360 nm in the H_2O layer was subtracted to determine the real absorbance value due to the hydrazone derivative of imidazole acetaldehyde.

Substrate inhibition: The activity decrease at high substrate concentration shown in Fig. 5 (a and b) may be due to substrate inhibition. The evident deviation from the straight line of the Lineweaver-Burk plots in the high substrate concentrations (Fig. 7) and the bell-type v-pS plots (Fig. 8) are unlikely to be due to some kind of alternation of the enzyme form, but rather due to substrate inhibition.

It might be considered that the concentration range (3×10^{-3} M, 10^{-2} M) at which substrate inhibition was observed is outside the range of physiological and pharmacological interest or also that the bell-

type v-pS curve shows just the optimal substrate concentration. However, in the case of copper-containing amine oxidases such as the hog kidney histaminase in the present study, it seems that the phenomenon of substrate inhibition is more significant relative to the enzyme-substrate interaction than just an artificial phenomenon outside the physiological concentration.

Several studies have reported substrate inhibition of copper-containing amine oxidases (7–9, 11–17). The K_s' values for histamine oxidation catalyzed by hog kidney histaminase were reported by Costa et al. (11) and Bardsley et al. (12), including the kinetic parameters K_m and V .

Our pS max of 3.2 was similar to that by Costa et al. ($K_m = 6.5 \times 10^{-5}$ M, pS = 3.2) (11). For cadaverine, they reported a pS max = 2.0 with $K_m = 7.3 \times 10^{-4}$ M (pH 7.4) (11). It was shown that the K_m and substrate concentration giving pS max for histamine were much lower than those for cadaverine, for which Costa et al. described weaker inhibition ($K_s' = 5.7 \times 10^{-2}$ M at pH 7.4) than that for histamine ($K_s' = 1.2 \times 10^{-2}$ M at pH 7.4) (11). Bardsley et al. (12) reported that the substrate inhibition was very weak for cadaverine.

The meaning of the K_s' depends upon the mechanism of substrate inhibition. According to the theory (7) used in the present study for calculation of the K_s' value, the enzyme-substrate complex is considered to react with a further substrate molecule to give an enzyme-bisubstrate complex, which results in inhibition at a high substrate concentration. K_s' is considered to be the dissociation constant for the enzyme-bisubstrate complex. A smaller K_s' value gives a larger substrate inhibitory effect.

Costa et al. suggested that substrate inhibition is caused by the attachment of a second substrate molecule to a negatively charged site, C, which prevents the substrate molecule from forming the usual enzyme-substrate complex (11). On the other hand, Bardsley et al. suggested that in addition to the negatively charged substrate binding site SBS (site C by Costa et al. (11)), there exists an inhibitory substrate-binding site, IBS, which has a strong affinity for the substrate because of its delocalized π -electron system

(12). The pH profiles of the kinetic parameters including K_s' were reported by Hill et al. and discussed with respect to the variation of substrate inhibition with pH (15).

For a more satisfactory explanation of substrate inhibition, in addition to more traditional kinetic studies employing a wide variety of substrates and studies of substrate specificity, we should also employ new techniques such as the spectroscopic technique which gives more direct information about the structural features of the active site on the enzyme molecule that is responsible for substrate inhibition.

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