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Fluorometric Studies on the Role of Calcium in Substrate Binding to 3-Ketovalidoxylamine A C–N Lyase

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In the present work, we studied the role of Ca^{2+} in the interaction between 3-ketovalidoxylamine A C–N lyase and its substrate in terms of the intrinsic tryptophan fluorescence of this enzyme. Intrinsic tryptophan fluorescence of the C–N lyase (1 μM), in the presence of endogenous Ca^{2+} (about 10 μM), was quenched by the addition of a substrate of the lyase, such as methyl-α-D-3-ketoglucoside or 3-ketotrehalose. The intensity of fluorescence returned to its original level in the absence of substrate when 0.1 mM ethylene glycol bis(2-aminoethylether)tetraacetic acid (EGTA) was added. In the presence of excess EGTA, however, the intrinsic fluorescence of the enzyme was unchanged by the addition of the substrates. The intensity of the fluorescence was also not changed by the addition of 0.1 mM $CaCl_2$ or EGTA alone. The substrate-dependent changes in the intrinsic fluorescence completely disappeared, showing a red shift of the emission maximum, by about 12 nm, at 0.4 M or more of guanidine·HCl. These experimental results suggest that Ca^{2+} has an important role in the binding of the substrate to the C–N lyase by maintaining the correct microenvironment and/or conformation around the tryptophan residue(s) of the enzyme molecule. The pH-dependent profiles of the K_d for the substrate binding to the lyase and C–O lyase activity indicate that the catalytic activity is not always correlated to the ability of the substrate to bind to the enzyme.

Keywords——3-ketovalidoxylamine A carbon–nitrogen lyase; carbon–oxygen lyase activity; tryptophan fluorescence; substrate binding; calcium effect; guanidine·hydrochloride effect; pH effect; *Flavobacterium saccharophilum*

Validamycin A, a major component of the validamycin complex produced by *Streptomyces hygroscopicus* var. *limoneus*, is extensively used as a curative fungicide for sheath blight of rice plants, as well as for soil-borne and other diseases caused by *Basidiomycetes*.[1,2] From the viewpoint of microbial clearance of fungicides, we attempted to elucidate the degradation processes of validamycins by *Flavobacterium saccharophilum* and found validamycin A to be hydrolyzed first to validoxylamine A by a β-glucosidase, followed by degradation to aminocyclitols and unsaturated ketocyclitols through 3-ketovalidoxylamine A by glucoside 3-dehydrogenase [EC 1.1.99.13] and 3-ketovalidoxylamine A C–N lyase.[3–5]

In a previous paper,[4] we reported the purification of 3-ketovalidoxylamine A C–N lyase from *F. saccharophilum*, and some of its properties. This enzyme catalyzes the cleavage of the C–N linkage of *p*-nitrophenyl-3-ketovalidamine to produce a *p*-nitroaniline and 5D-(5/6)-5-*C*-(hydroxymethyl)-2,6-dihydroxy-2-cyclohexen-1-one, but the enzyme also preferentially catalyzes the C–O linkage of *p*-nitrophenyl-α-D-3-ketoglucoside to produce a *p*-nitrophenol and 1,5-anhydro-D-*erythro*-hex-1-en-3-ulose.[4] The enzyme also has C–O lyase activity for methyl-α-D-3-ketoglucoside and 3-ketotrehalose produced from methyl-α-D-glucoside and trehalose by glucoside 3-dehydrogenase. Thus, it seems that the enzyme plays an important role in the sugar metabolism of *F. saccharophilum*.[6] Previous physicochemical studies of the enzyme indicated that its molecular weight and pI are 36000 and 10.5, respectively.[4] The enzyme

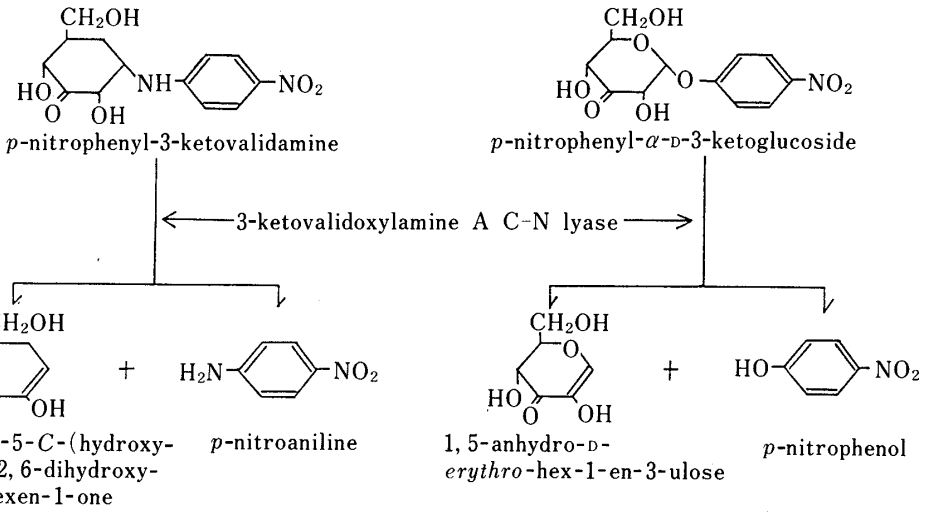

Chart 1. Enzymatic Degradation of Model Compounds by 3-Ketovalidoxylamine A C–N Lyase

contains five histidine, nine tyrosine, and four tryptophan residues per molecule of protein, and is inactivated by acylation of a single essential histidine residue with diethylpyrocarbonate.[7] Furthermore, the Ca^{2+} ion is an absolute requirement for enzyme activity.[4] The apparent dissociation constant and Hill coefficient of C–N lyase and Ca^{2+} ion were 0.19 μM and 1.6, respectively. The maximum activation of the enzyme was obtained at about 3 μM free Ca^{2+}.[4]

Intrinsic fluorescence in proteins has been the subject of many investigations.[8-10] Perturbation of the protein or enzyme emission spectra, as caused by addition of substrates or cofactors, provides a sensitive tool to investigate the microenvironment of the fluorescent amino acid residues, namely tryptophan and tyrosine.[11-15]

In the present paper, we wish to describe the role of Ca^{2+} in the interaction between 3-ketovalidoxylamine A C–N lyase and its substrate with reference to the effect of Ca^{2+} on the change of the fluorescence intensity emitted by C–N lyase upon binding with the substrate.

Materials and Methods

Enzyme Preparation——3-Ketovalidoxylamine A C–N lyase was purified from *F. saccharophilum* as described previously.[4] The enzyme preparation used in this study was homogeneous as shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein concentration was determined as described by Lowry *et al.*,[16] with bovine serum albumin as a standard.

Enzyme Assay——The C–O lyase activity of the 3-ketovalidoxylamine A C–N lyase was assayed using high-performance liquid chromatography, as described previously,[4] with *p*-nitrophenyl-α-D-3-ketoglucoside as a substrate.

Fluorescence Measurement——The fluorescence spectrum of the enzyme solution, in 0.1 M Tris–HCl buffer (pH 7.4), was measured with a Hitachi MPF-4 fluorescence spectrophotometer equipped with a circulating water bath at 25 °C, using excitation and emission wavelengths of 280 and 328 nm, respectively. In titration an appropriate volume of concentrated substrate solution (2—20 μl) was added to the enzyme solution (1 μM, 2.4 ml) in a thermostated quartz cell at 25 °C. The fluorescence intensity measured after each addition of the substrate was appropriately corrected for the dilution effect. The apparent dissociation constant (K_d) for the enzyme–substrate complex (ES) was obtained from a double-reciprocal plot of ΔF against substrate concentration according to the equation[17]

$$1/\Delta F = K_d/\Delta F_{max} \cdot 1/[S] + 1/\Delta F_{max}$$

where ΔF is the difference in fluorescence intensity in the absence and presence of substrate, ΔF_{max} is the maximum difference at infinite substrate concentration, and [S] is the concentration of the substrate added. In the present study, substrate concentrations used were greater than that of the enzyme (1 μM), and therefore it was considered that [S] could be used as the free substrate concentration in the calculation of K_d.

We have previously reported[4] the properties of C–N lyase using several *p*-nitrophenyl derivatives as substrates for the enzyme. In the present studies, methyl-α-D-3-ketoglucoside and 3-ketotrehalose were used as substrates instead of *p*-nitrophenyl derivatives, in order to eliminate the quenching of fluorescence due to the inner filter effect.

Determination of Calcium Concentration——A Nippon Jarrell Ash atomic absorption spectrophotometer, model AA-8500, was used to monitor the concentration of endogenous Ca^{2+} in the reaction mixture. When Ca^{2+} was not added to the reaction mixtures used, the concentration of endogenous Ca^{2+} in them was about 10 μM.

Chemicals——*p*-Nitrophenyl-α-D-glucoside, methyl-α-D-glucoside, and trehalose were obtained from Sigma Chemical Co. *p*-Nitrophenyl-α-D-3-ketoglucoside was prepared from *p*-nitrophenyl-α-D-glucoside by enzymatic dehydrogenation, according to the method described previously.[4] Methyl-α-D-3-ketoglucoside and 3-ketotrehalose were prepared from methyl-α-D-glucoside and trehalose by enzymatic dehydrogenation as reported.[7] Ethylene glycol bis(2-aminoethylether)tetraacetic acid (EGTA), guanidine·HCl, and all other chemicals used, were of the purest grade commercially available.

Results and Discussion

Fluorescence Properties

As shown in Fig. 1, the intrinsic fluorescence spectrum of the 3-ketovalidoxylamine A C–N lyase showed a typical tryptophan band, with excitation and emission maxima at 280 and 328 nm, respectively. The fluorescence intensity was strongly quenched by the addition of substrate, methyl-α-D-3-ketoglucoside, to the enzyme in the presence of about 10 μM Ca^{2+} (endogenous Ca^{2+} concentration in the reaction mixture). The fluorescence returned to its original intensity after EGTA was added (Fig. 2). As we have previously reported in studies of the Ca^{2+} requirement of the lyase, the maximum activation of the enzyme (0.2 μM) was obtained at about 3 μM Ca^{2+} (calculated from the K_d value for Ca^{2+}–EGTA complex).[4] Similar Ca^{2+}-dependent quenching was also observed on addition of 3-ketotrehalose as a substrate (data not shown). However, in the absence of these substrates, fluorescence was not affected by the addition of 0.1 mM $CaCl_2$ or 0.1 mM EGTA alone (Fig. 1). Mn^{2+}, a strong inhibitor of this lyase,[4] was less effective in quenching the fluorescence (data not shown). The products of this lyase reaction, methanol and D-glucose, did not appear to affect the intrinsic

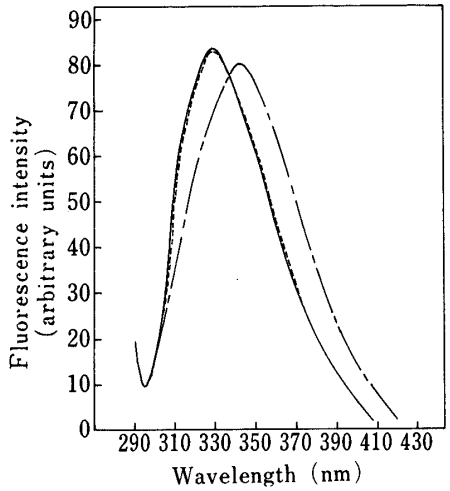

Fig. 1. Intrinsic Fluorescence Emission Spectrum of the Purified 3-Ketovalidoxylamine A C–N Lyase

Fluorescence spectra were measured at 25 °C in 0.1 M Tris–HCl buffer (pH 7.4), and the protein concentration was 1 μM. Fluorescence emission spectra were monitored with excitation at 280 nm. ——, C–N lyase alone; – – –, plus 0.1 mM EGTA (pH 7.4); —·—, plus 1 M guanidine·HCl (pH 7.4).

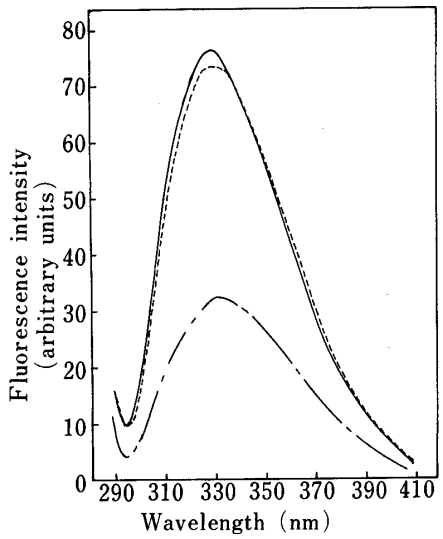

Fig. 2. Fluorescence Spectra of the C–N Lyase in the Absence and Presence of Methyl-α-D-3-ketoglucoside

Fluorescence emission spectra were monitored with excitation at 280 nm. ——, C–N lyase alone (the enzyme solution contained about 10 μM Ca^{2+} as determined by atomic absorption spectrophotometry); —·—, plus 40 μM methyl-α-D-3-ketoglucoside; ------, subsequent addition of 0.1 mM EGTA (pH 7.4).

enzyme fluorescence. Methyl-α-D-glucoside and trehalose did not affect the intrinsic fluorescence of the enzyme either. These results strongly suggest that these substrates can bind only to Ca^{2+}-bound enzyme molecules; and the subsequent binding induces conformational changes around the tryptophan residue(s) of the enzyme molecule.

Effect of Guanidine·HCl on Enzyme Activity and Intrinsic Fluorescence

Figure 3 shows the guanidine·HCl (pH 7.4) concentration dependences of the C–O lyase activity and intrinsic tryptophan fluorescence of the lyase. The enzyme activity toward *p*-nitrophenyl-α-D-3-ketoglucoside as a substrate was progressively decreased by increasing the concentration of guanidine·HCl. On the other hand, the guanidine·HCl-induced red shift of the fluorescence emission maximum (Figs. 1 and 3) was complete at about 0.4 M guanidine·HCl, which is similar to the concentration required for enzyme inactivation. Furthermore, Ca^{2+}-dependent fluorescence quenching by the substrate completely disappeared at about 0.4 M guanidine·HCl (data not shown). As has been previously reported,[18] the fluorescence intensity of *N*-acetyltryptophan ethylester was less affected by up to 1 M guanidine·HCl. According to our preliminary experiments, the C–O lyase activity was progressively inactivated by chemical modification with Koshland's reagent-I (water-soluble)

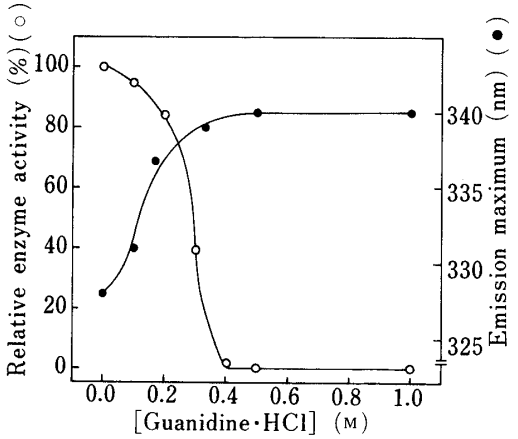

Fig. 3. Effect of Guanidine·HCl on Enzyme Activity and Intrinsic Fluorescence

Fluorescence emission spectral measurement and enzyme assay were carried out in 0.1 M Tris–HCl buffer (pH 7.4) containing 0 to 1.0 M guanidine·HCl (pH 7.4). Fluorescence emission spectra were monitored with excitation at 280 nm. The enzyme activity was measured as described in Materials and Methods. ○, relative enzyme activity; ●, fluorescence emission maximum.

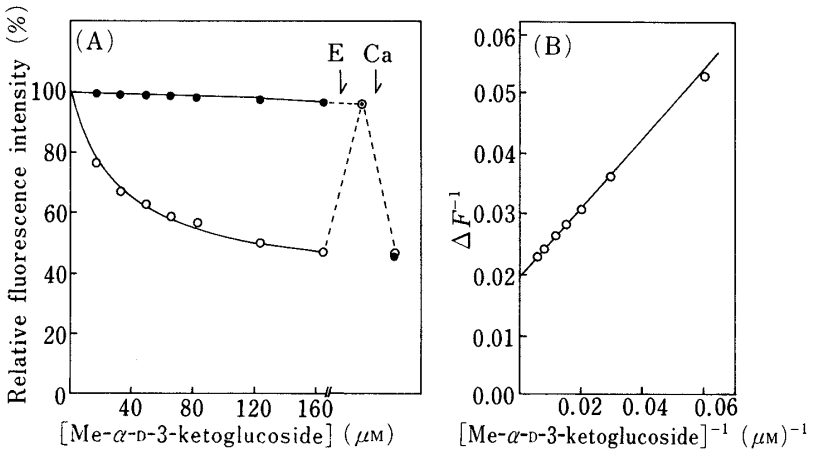

Fig. 4. Fluorometric Titration of the C–N Lyase with Methyl-α-D-3-ketoglucoside

(A) Fluorescence intensity is expressed relative to the value at no addition of methyl-α-D-3-ketoglucoside. Methyl-α-D-3-ketoglucoside was added in a stepwise manner up to 165 μM. After addition of 165 μM methyl-α-D-3-ketoglucoside, 0.1 M EGTA (pH 7.4) was added to give a final concentration of 0.1 mM. After addition of 0.1 mM EGTA, 0.2 and/or 0.3 M $CaCl_2$ was added to give a final concentration of about 0.2 and/or 0.3 mM. ○, in the presence of about 10 μM Ca^{2+}; ●, in the presence of 0.1 mM EGTA. E, addition of EGTA; Ca, addition of $CaCl_2$. (B) The double reciprocal plot of ΔF *versus* [S] using the data in (A).

(K-I W.S., Dojindo Lab.), which reacts specifically with tryptophan and slowly with cysteine residues of proteins (data not shown). Since the enzyme contains four tryptophan residues per mol of protein and no cysteine residues, it seems likely that one or more of the tryptophan residue(s) may be located close to the active center of the enzyme. However, this inactivation with K-I W.S. was not prevented by the substrates, methyl-α-D-3-ketoglucoside and 3-ketotrehalose (data not shown). Therefore, the conformation and/or microenvironment in the vicinity of the tryptophan residue(s) may be important for substrate binding to the enzyme and for lyase activity.

Fluorometric Titration of the C–N Lyase with Substrate

Figure 4A shows titration profiles for intrinsic fluorescence intensity with methyl-α-D-3-ketoglucoside in the presence and absence of Ca^{2+}. The fluorescence intensity was progressively quenched depending on the substrate concentration added, up to 165 μM, in the presence of about 10 μM Ca^{2+} (endogenous Ca^{2+} concentration in the reaction mixture), and returned to its original level on subsequent addition of 0.1 mM EGTA. Further addition of excess Ca^{2+} reversibly induced quenching. In the absence of Ca^{2+} (0.1 mM EGTA added), however, quenching of the fluorescence intensity did not occur even when up to 165 μM substrate was added.

As shown in Fig. 4B, the double-reciprocal plot of ΔF *versus* added substrate concentration ([S]) using the data in Fig. 4A was linear over the range of substrate concentration used. On the basis of these data, the apparent dissociation constant (K_d) for methyl-α-D-3-ketoglucoside–C–N lyase complex (ES) was calculated to be about 30 μM, according to the equation given under Materials and Methods.

Effect of pH on Substrate Binding to the C–N Lyase

As shown in Fig. 5, quenching of intrinsic fluorescence induced by addition of methyl-α-D-3-ketoglucoside at pH 5.0 was suppressed compared to that observed at pH 9.0. From the data of similar experiments at several pHs in the range of 4.5 to 9.0, the double reciprocal plot of ΔF *versus* [S] was linear for all cases (data not shown), as exemplified by the results at pH 7.4 (Fig. 4B). The apparent K_d values calculated from these data were plotted against pH (Fig. 6). A progressive decrease of the K_d value with a midpoint at about pH 6, roughly estimated

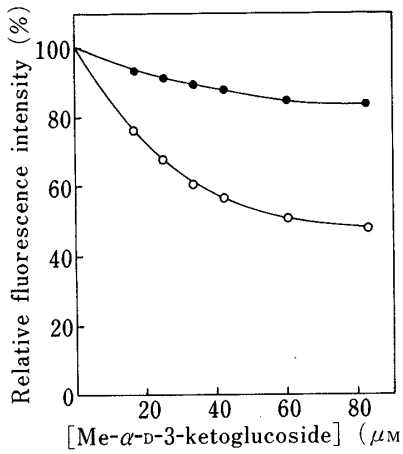

Fig. 5. Effect of pH on the Fluorometric Titration of the C–N Lyase with Substrate

Fluorescence intensity is expressed relative to the value at no addition of methyl-α-D-3-ketoglucoside. ●, pH 5.0; ○, pH 9.0.

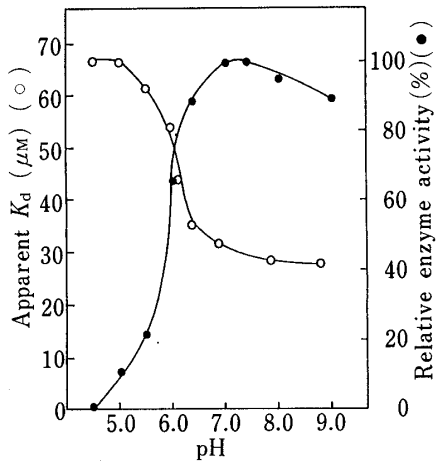

Fig. 6. Effect of pH on the Substrate Binding to the C–N Lyase

The apparent K_d values calculated from the fluorometric titration data were plotted against pH. The C–O lyase activity was measured as described in Materials and Methods. ○, apparent K_d values; ●, relative C–O lyase activity.

from the pH-dependence curve, was observed. Similar pH dependence was observed for the C–O lyase activity of this enzyme using *p*-nitrophenyl-α-D-3-ketoglucoside (Fig. 6). These results indicate that the lyase activity should be correlated to the apparent K_d value in general at pHs higher than 5.5 and pHs lower than 5.5; however, a correlation between them was not observed. Thus, it seems that the C–O lyase activity may be strongly suppressed by external factors, even though the substrate can bind to the enzyme with a K_d of about 70 μM at acidic pHs. This discrepancy between the catalytic activity of the lyase and the binding ability of its substrate was also observed in our preliminary experiments with the enzyme acylated by diethylpyrocarbonate (DEP). Modification of the lyase with DEP caused over 80% inhibition of enzyme activity, while the binding ability of the substrate to the DEP-modified enzyme under the same conditions was reduced by only about 50% in fluorometric experiments at pH 7.4 (data not shown). Therefore, it seems that inhibition of the lyase activity by acylation of histidine residue is not due solely to inhibition of substrate binding. Considering all these factors and our previous results,[7] a single histidine residue essential for the lyase activity may have a more important role in the catalytic processes than in enzyme–substrate binding. Further investigation is needed to elucidate the role of the histidine residue in substrate binding as well as in catalytic activity.

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