Chemical Modification by 2,4,6-Trinitrobenzenesulfonic Acid (TNBS) of an Essential Amino Group in 3-Ketovalidoxylamine A C–N Lyase

Masayoshi TAKEUCHI,[1] Ken-ichi NEYAZAKI and Katsuhiko MATSUI*

Department of Biochemistry, School of Pharmacy, Hokuriku University, Kanagawa-machi, Kanazawa, Ishikawa 920-11, Japan.
Received November 27, 1989

3-Ketovalidoxylamine A C–N lyase of *Flavobacterium saccharophilum* is a monomeric protein with a molecular weight of 36000, and contains 32 amino groups and no cysteine or cystine residues. The enzyme was inactivated by 2,4,6-trinitrobenzenesulfonic acid (TNBS) following pseudo-first order kinetics. Substrate of the lyase, *p*-nitrophenyl-3-ketovalidamine, protected the enzyme against the inactivation, suggesting that the modification occurred at or near the active site. Although several amino groups were modified by TNBS, a plot of log (reciprocal of the half-time of inactivation) *versus* log (concentration of TNBS) suggested that one amino group has an essential role in catalysis.

Keywords 3-ketovalidoxylamine A C–N lyase; amino group; chemical modification; 2,4,6-trinitrobenzenesulfonic acid; *Flavobacterium saccharophilum*

We recently found that the antifungal agent validamycin A is hydrolyzed first to validoxylamine A by a β-glucosidase, followed by degradation to validamine and valienamine through 3-ketovalidoxylamine A by D-glucoside 3-dehydrogenase [EC 1.1.99.13] and 3-ketovalidoxylamine A C–N lyase of *Flavobacterium saccharophilum*.[2-5] 3-Ketovalidoxylamine A C–N lyase catalyzed the cleavage of the C–N linkage in *p*-nitrophenyl-3-ketovalidamine and the C–O linkage in *p*-nitropheny α-D-3-ketoglucoside.[5,6]

In previous papers[5,7] we reported that 3-ketovalidoxylamine A C–N lyase of *F. saccharophilum* has a molecular weight of 36000 and optimum pH of 9.0, and that the enzyme contains a single histidine residue, which plays an essential role in the catalysis. However, the contributions of other amino acid residues to the active site of the enzyme have not been studied.

The present study describes the inactivation of the lyase by 2,4,6-trinitrobenzenesulfonic acid (TNBS), which is known to attack amino and sulfhydryl groups in peptides,[8] and proves the importance of an amino group for the activity of the enzyme.

Materials and Methods

Enzyme Preparation 3-Ketovalidoxylamine A C–N lyase was purified to a pure state according to the method of Takeuchi *et al.*[5] Protein was measured as described by Lowry *et al.*[9] with bovine serum albumin as a standard.

Enzyme Assay The activity of the 3-ketovalidoxylamine A C–N lyase was assayed using high-performance liquid chromatography, as described previously, with *p*-nitrophenyl-3-ketovalidamine as a substrate.[5]

Enzyme Modification with TNBS Chemical modification of the lyase by TNBS (Wako Pure Chemical) was carried out in the dark at 30 °C in a reaction mixture containing 0.25 M borate buffer (pH 9.5), 10% glycerol, the enzyme (3 μM) and different concentrations of TNBS solution, as indicated in the figure legends. The reaction was started by addition of TNBS solution, and aliquots of the modified enzyme were assayed for remaining activity.[5,7]

Estimation of Number of Amino Groups The number of amino groups modified by TNBS was calculated by the increase in absorbance at 367 nm, with a Union Giken high-sensitivity spectrophotometer SM 401, using an extinction coefficient for trinitrophenylated amine of $11000\,M^{-1}\cdot cm^{-1}$.[10]

Measurement of Circular Dichroism (CD) Spectrum For circular dichroism measurement, the reaction was stopped by gel filtration of the incubation mixture on a Sephadex G-25 column (1.4 × 10 cm) previously equilibrated with 10 mM borate buffer (pH 9.5) containing 10% glycerol. CD spectra were measured in a 10 mm light-path cell with a Jasco J-20A spectropolarimeter at room temperature. In the calculation of residue molar concentration, an average residue weight of 107.5 was used.

Results and Discussion

When the enzyme was incubated with varying concentrations of TNBS at pH 9.5, a time-dependent inactivation occurred following pseudo-first order kinetics as shown in Fig. 1A. Figure 1B shows the effect of TNBS concentration on the apparent pseudo-first order rate constant of inactivation, which was estimated from the data in Fig. 1A. This constant was proportional to the reagent concentration, indicating that the reaction between the enzyme and the reagent is a simple bimolecular reaction. From these data, a second order rate constant of $6.0\,M^{-1}\cdot min^{-1}$ was obtained. The value is consistent with that for bovine pancreatic deoxyribonuclease A ($7\,M^{-1}\cdot min^{-1}$ at pH 9.5).[10] The CD spectra (from 200 to 250 nm) with native and TNBS-modified (80%-inactive enzyme) enzymes had very similar shapes (data not shown). Thus, TNBS modification of the lyase had a marked effect on the enzymatic activity, but not on the conformation of the enzyme. The inactivation of the lyase by TNBS was protected by the substrate, but not by the *p*-nitrophenylvalidamine (Fig. 2). This result suggests that the modification occurred at or near the active site. It is well known that TNBS reacts with amino and sulfhydryl groups in proteins.[8] The enzyme contains 32 lysine residues per molecule of protein, but no cysteine or cystine residues.[7] In addition, the absorption spectrum of TNBS-modified enzyme showed maxima at 345 and 420 nm, characteristic of a trinitrophenylated amino group[8] (data not shown). Thus the inactivation of the enzyme by TNBS is apparently due to the modification of amino groups.

The number of modified amino groups can usually be estimated by measuring the absorbance change at 367 nm ($\varepsilon = 11000\,M^{-1}\cdot cm^{-1}$) upon the addition of TNBS.[10] By this method, we examined the relationship between the number of modified amino groups and the residual activity. As shown in Fig. 3, the fast initial drop of lyase activity correlates with the modification of 8 amino groups. The modification of 12 amino groups per molecule of enzyme resulted in a complete inactivation of the lyase. The apparent K_m value (0.24 mM) obtained from 80%-inactive enzyme was the same as that from the native enzyme (data not shown). To determine the number of reactive amino groups at the active site of the enzyme, a plot of log (reciprocal of the half-time of inactivation) *versus* log

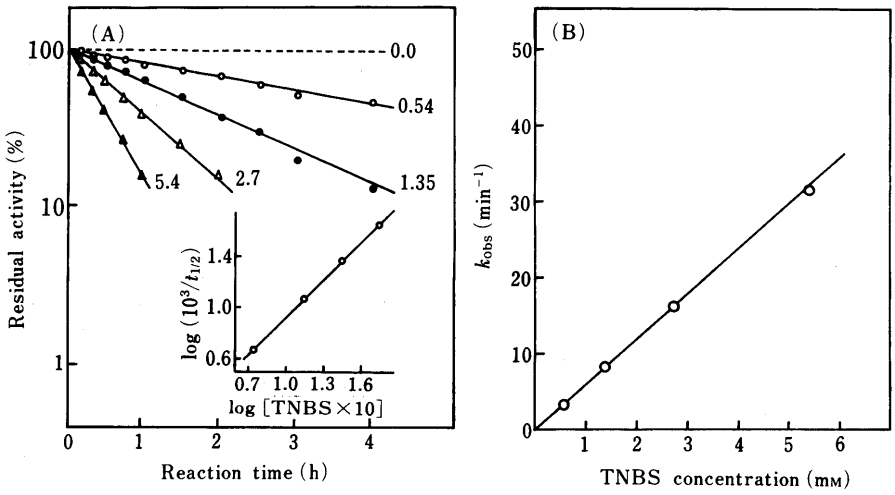

Fig. 1. Kinetics of Inactivation of 3-Ketovalidoxylamine A C–N Lyase by TNBS

(A) The enzyme (3 μM) was incubated with indicated concentrations of the reagent, and the activity was assayed as described in Materials and Methods. Numerals indicate TNBS concentration in mM. Inset: apparent order of the inactivation process with respect to reagent concentration. The half-times for inactivation were obtained from the results in Fig. 1A. (B) Effect of TNBS concentrations on the inactivation rate constant. The pseudo-first order rate constant was obtained from the data in (A) according to the equation: $\ln(V_t/V_0) = -k_{obs} \cdot t$ where V_0 and V_t show the enzyme activity at time 0 and t, respectively.

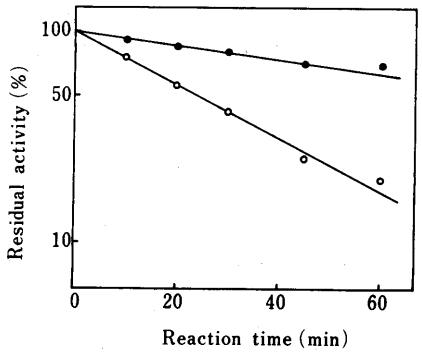

Fig. 2. Protective Effect of the Substrate against TNBS-Inactivation of 3-Ketovalidoxylamine A C–N Lyase

The enzyme (3 μM) was incubated with 5.4 mM TNBS in the presence (●) and absence (○) of 1.0 mM *p*-nitrophenyl-3-ketovalidamine. Small aliquots were withdrawn at indicated times, and the enzyme activity was measured.

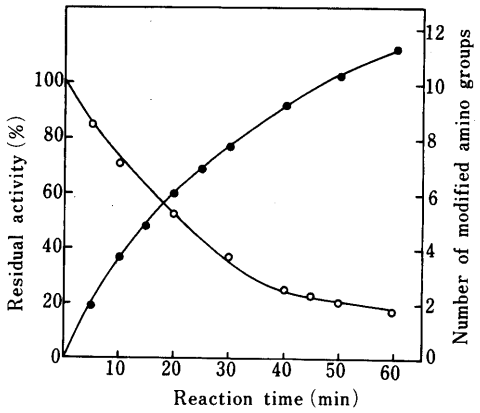

Fig. 3. Correlation Between the Enzyme Activity and the Number of Modified Amino Groups

The enzyme (3 μM) was incubated with 5.4 mM TNBS. Enzyme activity (○) was followed by the standard assay. Modification of amino groups (●) was calculated from the increase of absorbance at 367 nm using an excitation coefficient of 11000 $M^{-1} \cdot cm^{-1}$.

(concentration of TNBS) was used. Such a plot should be a straight line with slope n, where n is the number of inhibitor molecules reacting with the active site of the enzyme to form an enzyme-inhibitor complex.[11] As shown in the Fig. 1A inset, the plot was a straight line with a slope of 1.0. Although several amino groups were modified by TNBS, this double logarithmic plot suggested that one of these groups has an essential role in catalysis. Further investigation is necessary to elucidate the role of the amino groups (α- or ε-amino group) as well as the other amino acid residues.

References and Notes

1) Present address: *Fuji Chemical Industries, Ltd., 530 Chokeiji, Takaoka, Toyama 933, Japan.*
2) N. Asano, M. Takeuchi, K. Ninomiya, Y. Kameda and K. Matsui, *J. Antibiot.*, **37**, 859 (1984).
3) M. Takeuchi, K. Ninomiya, K. Kawabata, N. Asano, Y. Kameda and K. Matsui, *J. Biochem.* (Tokyo), **100**, 1049 (1986).
4) M. Takeuchi, N. Asano, Y. Kameda and K. Matsui, *Agric. Biol. Chem.*, **52**, 1905 (1988).
5) M. Takeuchi, N. Asano, Y. Kameda and K. Matsui, *J. Biochem.* (Tokyo), **98**, 1631 (1985).
6) M. Takeuchi, N. Asano, Y. Kameda and K. Matsui, *Chem. Pharm. Bull.*, **36**, 3540 (1988).
7) M. Takeuchi, N. Asano, Y. Kameda and K. Matsui, *J. Biochem.* (Tokyo), **99**, 1571 (1986).
8) T. Okuyama and K. Satake, *J. Biochem.* (Tokyo), **47**, 454 (1960).
9) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
10) B. V. Plapp, S. Moore and W. H. Stein, *J. Biol. Chem.*, **246**, 939 (1971).
11) H. M. Levy, P. D. Leber and E. M. Ryan, *J. Biol. Chem.*, **238**, 3654 (1963).