MODIFICATION OF AMINO GROUPS IN *CANDIDA UTILIS* URICASE WITH NAPHTHOQUINONE DISULFONIC ACID IN RELATION TO THE ENZYMIC ACTIVITY

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1. Introduction

Uricase from *Candida utilis* had neither metal ions such as Cu^{2+} and Fe^{3+} nor cofactors and cysteine residues in the molecule did not participate in enzymic activity [1]. Uricases from *Bacillus fastidiosus* and from *Aspergillus flavus* did not contain metal ions and cofactors [2,3]. Although uricase has been believed to be one of the metallo- [4,5] and/or sulfhydryl-enzymes [4,6–8] and a mechanism of the enzymic reaction has been proposed [5], the biochemical and physicochemical properties of uricase still remain to be clarified.

Here, amino groups in uricase from *Candida utilis* were modified with β-naphthoquinone-4,6-disulfonic acid (NQDS) after oxidation of cysteine residues in uricase with *o*-iodosobenzoate. Approximately 65 out of the total 96 amino groups in the uricase molecule were accessible to the reagent and the remaining 31 groups became accessible after denaturation. Two out of 24 amino groups in a subunit molecule reacted preferentially with the reagent accompanied by a dramatic loss of enzymic activity. Such a loss was not observed in the presence of xanthine, one of the substrate analogues. These results suggested the participation of two amino groups in the subunit molecule in the enzymic activity. The modification with other reagents, such as acetic anhydride, did not cause a comparable loss of enzymic activity.

2. Materials and methods

The partially purified uricase (4.3 units/mg protein) from *Candida utilis* was kindly supplied by Toyobo Co. Ltd. The crude preparation of uricase was further purified by affinity chromatography with xanthine–agarose conjugate and by gel chromatography with Sephadex G-200 as in [1]. The highly purified uricase (M_r 120 000) has spec. act. 25.8 units/mg protein and is composed of 4 identical subunits with 30 000 M_r. The molar extinction coefficient was 2.03×10^5 M^{-1} cm^{-1} at 280 nm, assuming 120 000 M_r and $A^{1\%}_{280}$ = 16.9 [1]. The enzymic activity of uricase was determined by measuring the absorbance decrease of uric acid at 293 nm with a Cary recording spectrophotometer model 14 [1]. The molar extinction coefficient of uric acid was 1.26×10^4 M^{-1} cm^{-1} at 293 nm [9].

β-Naphthoquinone-4,6-disulfonic acid was obtained from Seikagaku Kogyo Co. The modification of amino groups in uricase was done as in [10]. The reagent has the ability to react specifically with ε-amino groups of lysine, N-terminal amino acid as well as with bound cysteine residues. The total number of amino groups and cysteine residues in the uricase molecule were 96 and 12, respectively and were determined by amino acid analysis and by titration with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) [1]. To prevent the modification of cysteine residues with NQDS, the sulfhydryl groups in uricase were oxidized to sulfenic acid residues with *o*-iodosobenzoate prior to modification with NQDS. Oxidized uricase (IB-uricase) retained the enzymic activity of 21.4 units/mg protein. The attachment of a bulky group to the sulfhydryl residues in uricase by modifying reagents, such as DTNB and *p*-chloromercuribenzoate instead of by simple oxidation, caused a loss of enzymic activity [1], due to steric hindrance of the modifier with regard to the formation of the enzyme–substrate complex. Therefore, IB-uricase was prepared as in [1] and subjected to modification of the amino groups in uricase with NQDS, as follows:

Abbreviations: NQDS, β-naphthoquinone-4,6-disulfonic acid; IB-uricase, *o*-iodosobenzoate-oxidized uricase

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To 500 μl IB-uricase (1.1–18.2 μM) in 0.1 M phosphate buffer (pH 9.0) was added 500 μl of NQDS (20 μM–23 mM) dissolved in 0.1 M phosphate buffer (pH 9.0) in the presence or the absence of xanthine (1 mM). The reaction mixture was incubated for 2.5 h at 25°C to complete the reaction. The number of amino groups modified with NQDS was spectrophotometrically determined by measuring the absorbance at 480 nm after the addition of 750 μl Triton X-100 solution (3.3%) in 33 mM acetic acid to the sample solution (400 μl). Molar extinction coefficient of the amino group modified with the reagent was 4.01×10^3 M^{-1} cm^{-1} at 480 nm [10]. Uricase denatured by alkali was prepared by incubation of IB-uricase solution (12.7 μM) with 0.5 M NaOH for 3 h at room temperature. The analysis of amino acid composition of modified IB-uricase was performed with an amino acid analyzer model JLC-6AH after reduction with 2-mercaptoethanol followed by carboxymethylation.

3. Results and discussion

Fig.1 shows the reaction curves obtained by the modification of IB-uricase (curves A,B) and denatured uricase (curve C) with the various concentrations of NQDS together with the change of the enzymic activities by the modifications (curves D,E). The degree of modification of amino groups in alkaline denatured IB-uricase was enhanced by increasing NQDS concentration and tended to approach a constant level at >10 mM NQDS (curve C). The maximum number of amino groups modified was ~96 which is in good agreement with that obtained by amino acid analysis [1]. In the case of native IB-uricase (curve A), the reactivity of amino groups was also enhanced by increasing NQDS concentration and at >9.0 mM the reaction curve remained at a constant level corresponding to ~65 amino groups. This indicates that 65 out of the total 96 amino groups in the uricase molecule are accessible to the reagent and the remaining 31 groups are not accessible probably due to the location of these amino groups in the interior of the protein molecule. The change of the enzymic activity of IB-uricase by the modification is shown by curve D. Dramatic reduction of enzymic activity was observed by the modification of amino groups only with 230 μM NQDS.

A similar experiment was carried out for the modification of IB-uricase with the same reagent in the presence of xanthine which is one of the substrate analogues of uricase. The reaction curve obtained in the presence of xanthine (curve B) was almost the

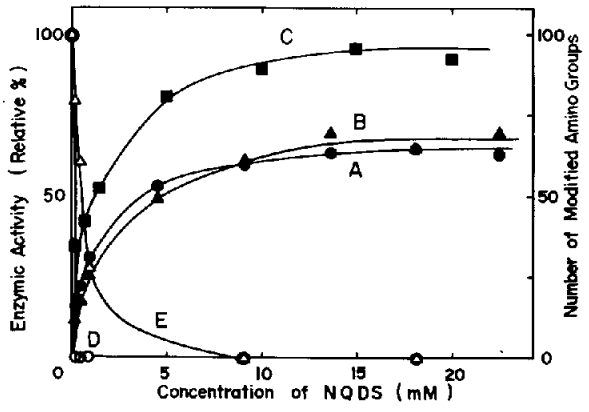

Fig.1. Modification of IB-uricase (1.7 μM) or denatured IB-uricase (1.1 μM) by various concentrations of NQDS (230 μM–23 mM) and change of enzymic activity: (A,B) modification of IB-uricase by the reagent in the absence (•) and the presence of 1 mM xanthine (▲), respectively; (C) modification of denatured uricase by the reagent (■); (D,E) change of enzymic activities in the absence (○) and presence (△) of 1 mM xanthine, respectively.

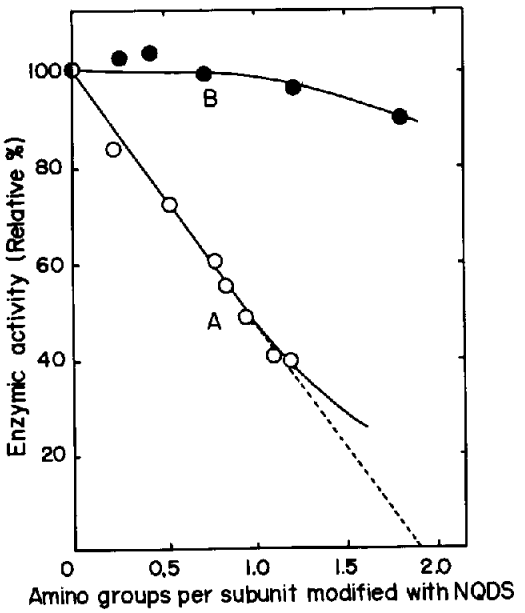

Fig.2. Plot of the enzymic activity of modified IB-uricase *vs* the number of amino groups modified with NQDS in the subunit molecule: (A,B) in the absence (○) and the presence (•) of xanthine, respectively.

Table 1
Modification of IB-uricase with NQDS

No. amino groups/ subunit	No. carboxymethyl cysteine/subunit[a]
0	2.7
2.0	2.7
3.2	2.7
4.3	2.6
5.3	2.7

[a] NQDS-modified IB-uricases were reduced and carboxymethylated in the presence of 6 M guanidine and subjected to amino acid analysis

same as that in the absence of xanthine (curve A). On the other hand, the enzymic activity of IB-uricase modified in the presence of xanthine decreased slowly by the modification and was completely lost at 9 mM NQDS. These results imply that xanthine with $K_i = 1.2 \times 10^{-5}$ M [5] prevents the modification of the amino group(s) located in the active center of the uricase molecule.

In order to clarify the relationship between the enzymic activity and the degree of modification of IB-uricase with lower concentrations of NQDS (120–200 μM), the next series of experiment was carried out in the presence and the absence of xanthine. Fig.2 shows the plotting of the enzymic activity of modified uricase against the number of amino groups modified/subunit molecule. The enzymic activity decreased linearly by the modification of amino groups in the absence of xanthine (curve A). The extrapolation of the linear plot intersected at a point on the horizontal axis, where only two amino groups in the subunit molecule were modified by NQDS. In the presence of xanthine the enzymic activity decreased slowly and retained 80% of the original activity. These results suggest that two amino groups in the subunit are located at the active site and closely associated with the enzymic activity.

To see whether cysteine residues of IB-uricase were modified with NQDS or not, NQDS-modified IB-uricase was reduced with 2-mercaptoethanol followed by carboxymethylation and the number of carboxymethylated cysteine residues was determined. The result is shown in table 1. Approximately 3 carboxymethylated cysteines in a subunit were observed and did not change even when the number of modified amino groups in uricase increased. This indicates that modification with NQDS affects only amino groups and not cysteine residues oxidized with *o*-iodosobenzoate.

Other amino acids in uricase, except lysine, were not modified by NQDS.

Similar modification studies on uricase were carried out with other reagents, such as acetic anhydride [11], trinitrobenzene sulfonate [12] and 2-methoxy-5-nitrotropone [13]. However, no dramatic reduction of enzymic activity was observed by the modification of amino groups in uricase with such reagents. It is noteworthy that the reagent, NQDS, reacts preferentially with the amino groups located at the active center of uricase, in contrast to other reagents.

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