Nicotinamide Adenine Dinucleotide Phosphate-specific Glutamate Dehydrogenase of *Neurospora*

III. INACTIVATION BY NITRATION OF A TYROSINE RESIDUE INVOLVED IN COENZYME BINDING*

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*Neurospora* glutamate dehydrogenase (NADP-specific) is rapidly inactivated upon reaction with tetranitromethane. This inactivation is completely prevented by the presence of coenzyme (NADP) or nicotinamide mononucleotide (NMN) but not by substrate, NADH, or 2'-monophosphoadenosine-5'-diphosphoribose. Amino acid analysis indicates that the primary effect of modification is nitration of a single residue of tyrosine per polypeptide chain. We have identified the reactive tyrosine by isolation of a single, uniquely labeled peptide after hydrolysis with trypsin followed by cleavage with cyanogen bromide. The modified residue proved to be tyrosine-168 in the linear sequence. This residue is not present in the part of the sequence that had been previously implicated as involved in the binding of the adenylate portion of the coenzyme.

Both NMN and 2-monophosphoadenosine-5'-diphosphoribose act as competitive inhibitors of NADP in the oxidation of glutamate with *K*ₜ values of 4.65 × 10⁻⁴ M and 4.30 × 10⁻⁴ M, respectively. Thus, the specific protection afforded by NADP and NMN, but not by 2'-monophosphoadenosine-5'-diphosphoribose, indicates that tyrosine-168 is involved in binding the nicotinamide portion of the coenzyme.

The NADP-dependent glutamate dehydrogenase (EC 1.4.1.4) of *Neurospora crassa* is a hexameric protein containing identical subunits of molecular weight 48,656. The primary structure of this enzyme has recently been reported (1), and it has been demonstrated that portions of the sequence are homologous with those of the bovine and chicken glutamate dehydrogenases previously described (1,2). The active site lysine, reactive with pyridoxal 5'-phosphate and N-ethylmaleimide (3), is residue 113 in the sequence of the *Neurospora* enzyme (1).

It has been reported that binding of coenzyme to bovine glutamate dehydrogenase results in perturbation of certain enzyme chromophores; specifically, both a red-shifted tryptophan and a blue-shifted tyrosine appear to contribute to the absorption spectrum (4). Furthermore, it has been suggested that stacking of the nicotinamide moiety of the coenzyme with some aromatic side chain of the enzyme might be involved in energy transfer (4). Although similar spectrophotometric data are not yet available for the *Neurospora* enzyme, the fact that the two proteins display considerable sequence homology suggested the desirability of investigating the role of tyrosine residues in the *Neurospora* enzyme. In this paper we report the inactivation of the enzyme by reaction with tetranitromethane and the identification of a single tyrosine residue as the site of nitration.

EXPERIMENTAL PROCEDURES

**Materials**—*Neurospora* glutamate dehydrogenase (NADP) was purified as described previously (3). Trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone was purchased from Worthington and tetranitromethane from Aldrich. NADH, NADP(H), α-ketoglutarate, and iodoacetamide were products of Calbiochem. 2'-Monophosphoadenosine-5'-diphosphoribose (the analog of NADP lacking the nicotinamide ring) was the generous gift of Dr. Nathan K. Kaplan, University of California at San Diego. Cyanogen bromide was a product of the Pierce Chemical Co. and guanidine hydrochloride ("ultrapure") was purchased from Schwarz/Mann. Nicotinamide mononucleotide and N-methyl nicotinamide were gifts of Dr. David Sigman of this department.

**Reaction with Tetranitromethane**—Inactivation of the enzyme was initiated by addition of the desired excess of C(NO₂)₂ (as a 0.084 M solution in absolute ethanol) to a solution of the enzyme (1.0 mg/ml) at 30° in 0.05 M Tris-acetate buffer at pH 8.0. Reactions were terminated at the appropriate time by addition of 2-mercaptoethanol in 5-fold excess over C(NO₂)₂, followed by adjustment of the pH to 4.5 and dialysis against 5% acetic acid prior to amino acid analysis.

For preparative purposes, 2.0 μmol of the enzyme were inactivated as described above, by incubation with 84 μmol of C(NO₂)₂ giving a 2.67-fold excess of reagent over tyrosine residues. The nitrated protein, after dialysis, was taken to dryness, redissolved in 0.3 M Tris-HCl at pH 8.6 containing 6% guanidinium HCl and 0.2% EDTA, and reduced under nitrogen at 55° by the addition of 100 μl of mercaptoethanol. After

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1 The abbreviations used are: C(NO₂)₂, tetranitromethane; A(2'-P)RPRR, 2'-monophosphoadenosine-5'-diphosphoribose; Rapp, mobility relative to aspartic acid in paper electrophoresis at pH 1.0.
RESULTS

Reaction with Tetranitromethane—The course of the reaction of C(NO$_3$)$_3$ (8.4 x 10$^{-4}$ M) with the enzyme (1.1 mg/ml) at 30$^\circ$ in 0.05 M Tris-acetate buffer at pH 8.0, is shown in Fig. 1. The activity of aliquots of the reaction mixture, taken at increasing times of reaction, was determined in the standard reductive amination assay (10). As shown in the inset, the loss of enzyme activity is a pseudo-first order process with a half time of approximately 2 min under the experimental conditions. Loss of activity was essentially complete after 10 min.

Amino acid analysis of the eluted material indicated that the nitrotyrosine-containing fraction was not completely homogeneous and contained methionine in addition to nitrotyrosine. Accordingly, the material was cleaved with cyanogen bromide (7) and the resulting mixture was repurified by preparative electrophoresis at pH 1.9. Amino acid analysis of the eluted material indicated that the nitrotyrosine-containing fraction was not completely homogeneous and contained methionine in addition to nitrotyrosine. Accordingly, the material was cleaved with cyanogen bromide (7) and the resulting mixture was repurified by preparative electrophoresis at pH 1.9.

Amino Acid Analysis—Samples were hydrolyzed for 22 hours at 110$^\circ$ in evacuated glass tubes containing 1 ml of 6 N HCl and a drop of 1% phenol in water. Analyses were performed with a Spinco model 120B amino acid analyzer (8). 3-Nitrotyrosine was identified by its elution time in this system (9) and estimated with the ninhydrin color value for tyrosine (8).

Protection of Enzyme Activity—Treatment of the enzyme with C(NO$_3$)$_3$, in the presence of 10 mM $\alpha$-ketoglutarate failed to protect the enzyme activity (Fig. 2). However, when the reaction with C(NO$_3$)$_3$ was run in the presence of 5 mM NADP or NADPH, the enzyme activity was unaffected even when a 30- to 50-fold molar excess (relative to total tyrosine content) of NADP$_3$ was employed. Moreover, there was no nitration of tyrosine in the presence of NADP under the same conditions as in the absence of coenzyme when 1 nitrotyrosine residue was formed. NAD or NADH did not prevent inactivation by C(NO$_3$)$_3$, indicating that protection by NADP or NADPH was specific for the coenzyme. Low levels of NADP(H) (0.1 mM) afforded partial protection against inactivation by C(NO$_3$)$_3$ (data not shown).

In order to investigate which portion of the coenzyme molecule might be responsible for the protective effect, the nitration reaction was performed in the presence of the 2'-phosphate ester of adenosine diphosphate ribose at 5 mM concentration. As shown in Fig. 2, this compound did not prevent inactivation of the enzyme by C(NO$_3$)$_3$, thereby suggesting that the nicotinamide moiety of the coenzyme may be involved in the protection by NADP(H).

The involvement of the nicotinamide mononucleotide moiety was clearly demonstrated by performing the reaction with C(NO$_3$)$_3$, in the presence of 10 mM or 0.1 mM nicotinamide...
mononucleotide (reduced form). As shown in Fig. 2, the presence of 10 mM NMNH completely protected the enzyme activity against C(NO₂)₄. At a concentration of 0.1 mM NMNH, partial protection was obtained. N-Methyl nicotinamide (10 mM) was without effect on the rate of inactivation.

Tests with NMNH demonstrated that it could not substitute for NADP as a cofactor for the reductive amination reaction. However, NMN proved to be an inhibitor when included in the oxidative deamination substrate solution at 0.01 to 0.10 mM. Fig. 3 shows that both A(2'-P)RPPR and NMN are inhibitors of the enzyme. Within experimental error, both substances are competitive with NADP in the oxidation of glutamate. The determined Kᵢ values are 4.30 x 10⁻⁴ m for A(2'-P)RPPR and 4.60 x 10⁻⁴ m for NMN, as compared to Kᵢ = 5.6 x 10⁻⁴ m for NADP.

Molecular Weight of Nitrated Glutamate Dehydrogenase

The effect of nitration upon the subunit molecular weight was determined by sodium dodecyl sulfate gel electrophoresis with mercaptoethanol or without. The subunit molecular weight was estimated by comparison with a standard curve prepared with suitable standards of known molecular weight. The subunit molecular weight, both in the presence and absence of mercaptoethanol, was approximately 50,000 and identical with that of the unmodified enzyme, thus indicating that no cross linking of the subunits had occurred.

Identification of Reactive Tyrosine—Treatment of 2 μmol of enzyme with C(NO₂)₄ followed by carboxamidomethylation, gave a preparation containing 1.1 residues of 3-nitrotyrosine per subunit. After hydrolysis with trypsin, the two resulting fragments, Peptides CN-1 and CN-2 (Table I), were isolated by paper electrophoresis at pH 1.9. The determined Kᵢ values are 4.30 x 10⁻⁴ m for A(2'-P)RPPR and 4.60 x 10⁻⁴ m for NMN, as compared to Kᵢ = 5.6 x 10⁻⁴ m for NADP.

In order to split the peptide at the unique residue of methionine, it was dissolved in 70% formic acid and treated with 1.25-fold molar excess of CNBr. The two resulting peptides were isolated by paper electrophoresis at pH 1.9. After elution from the paper, amino acid analysis indicated that at least five components were present; however, only one (Rₐₐₐ = 0.75) turned yellow when sprayed with 10% NH₂OH. After elution from the paper, amino acid analysis indicated that although the peptide was not completely homogeneous, it undoubtedly represented residues 160 through 170 (Fig. 5), since it contained equimolar amounts of phenylalanine, methionine, tyrosine, and 3-nitrotyrosine. Only the tryptic peptide representing this segment of the protein would be expected to contain these residues. Inasmuch as this portion of the sequence contains 2 residues of tyrosine, the site of modification could not be identified on the basis of the composition alone.

In order to split the peptide at the unique residue of methionine, it was dissolved in 70% formic acid and treated with a 50-fold molar excess of CNBr. The two resulting peptides were isolated by paper electrophoresis at pH 1.9. After elution from the paper, amino acid analysis indicated that although the peptide was not completely homogeneous, it undoubtedly represented residues 160 through 170 (Fig. 5), since it contained equimolar amounts of phenylalanine, methionine, tyrosine, and 3-nitrotyrosine. Only the tryptic peptide representing this segment of the protein would be expected to contain these residues. Inasmuch as this portion of the sequence contains 2 residues of tyrosine, the site of modification could not be identified on the basis of the composition alone.

Although NMNH was added as the ligand in this experiment, it seems highly likely that it was at least partially oxidized by the addition of C(NO₂)₄.
suggestions (11, 12) that residues 219 through 254 of the moiety of the coenzyme. This would be in accord with earlier that tyrosine-168 is not involved in binding the adenylate pheoadenosine-5’.diphosphoribose does not protect indicates by showing that NAD or NADH is without effect on the rate of NADPH. The specificity of this protection was demonstrated by showing that NAD by the coenzyme binding site resulting from nitration.

**DISCUSSION**

*Neurospora* glutamate dehydrogenase (NADP-specific) has been shown to be inactivated by reaction with tetranitromethane concomitant with the nitration of a single residue of tyrosine per polypeptide chain. Nitration did not cause cross-linking of the subunits. Although amino acid analysis indicated that some oxidation of cysteine to cystic acid had occurred, the oxidation of cysteine cannot be the cause of the inactivation since we had shown earlier (3) that sulfhydryl groups are not involved in the activity of this enzyme.

The reactive tyrosine has been identified as number 168 in the amino acid sequence of the subunit. This residue is intermediate in position between the active site lysine (residue 113 (1, 3)) and the portion of the sequence, residues 219 through 254, that has been implicated in binding the adenylate moiety of the coenzyme based on the homology of the glutamate dehydrogenases with other dehydrogenases of known conformation (11, 12).

Enzyme activity could not be protected by substrate, but inactivation by tetranitromethane and nitration was completely prevented by the presence of the coenzyme NADP or NADPH. The specificity of this protection was demonstrated by showing that NAD or NADH is without effect on the rate of inactivation. Furthermore, the finding that 2’-monophosphoadenosine-5’-diphosphoribose does not protect indicates that tyrosine-168 is not involved in binding the adenylate moiety of the coenzyme. This would be in accord with earlier suggestions (11, 12) that residues 219 through 254 of the *Neurospora* enzyme probably comprise at least a portion of the adenylate binding site. The finding that nitration of tyrosine-168 does not occur in the presence of 10 mM NMN(H) indicates that this residue is in some way involved in interaction with the nicotinamide portion of the coenzyme, although our data do not distinguish between interaction of tyrosine-168 directly with NADP(H) and a conformational change in the coenzyme binding site resulting from nitration.

The failure of NAD(H) to protect the enzyme from nitration despite its containing a nicotinamide moiety indicates that the absence of the 2’-phosphate group on the adenosine portion of NAD precludes any binding with the enzyme. Indeed, it suggests that there is actual repulsion of NAD by the coenzyme site of the *Neurospora* enzyme. Not only does NAD not function as a cofactor but it does not act as an inhibitor of the enzyme.

Fisher (4, 13) has shown that binding of coenzyme to bovine glutamate dehydrogenase causes perturbations in the absorption spectra of both the tyrosine and tryptophan chromophores and it has been suggested that a tryptophanyl residue may be involved in energy transfer between enzyme and cofactor (14), with the nicotinamide ring stacking with the aromatic side chain. It is interesting to note that the reactive tyrosine is a conserved residue in comparing the bovine and *Neurospora* glutamate dehydrogenases, being found at residues 183 and 168, respectively, in the two enzymes (Fig. 5). Furthermore, although there is very little homology between the vertebrate and *Neurospora* enzymes in the 11 residues following tyrosine-168, strong homology is found in the region between residues 143 and 168. 

Although tyrosine-168 is conserved in the bovine enzyme, no direct information is available on its possible role in maintenance of the activity. Nitration of bovine glutamate dehydrogenase results in rapid desensitization to the allosteric inhibitor GTP concomitant with nitration of tyrosine-406 (15); more extensive nitration results in a slower loss of enzyme activity with nitration of a number of additional residues (15). Thus, it is presently impossible to say whether the inactivation is due to nitration of tyrosine-183 (of the bovine enzyme) or to other factors. Although a tyrosine residue may well be involved in coenzyme binding by the bovine enzyme, its precise identification is not possible at this time.

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**REFERENCES**


* K. M. Blumenthal and E. L. Smith, unpublished data.
Nicotinamide adenine dinucleotide phosphate-specific glutamate dehydrogenase of Neurospora. III. Inactivation by nitration of a tyrosine residue involved in coenzyme binding.

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