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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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 for the two proteins display considerable sequence homology (3), the identification of a single tyrosine residue as the site of 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 NAD and 2-monophospho-dinosine-5'-diphosphoribose act as competitive inhibitors of NADP in the oxidation of glutamate with $K_I$ values of $4.60 \times 10^{-4}$ M and $4.30 \times 10^{-4}$ M, respectively. Thus, the specific protection afforded by NADP and NMM, but not by 2'-monophospho-dinosine-5'-diphosphoribose, indicates that tyrosine-168 is involved in binding the nicotinamide portion of the coenzyme.

The 4.3-fold excess of reagent over tyrosine residues. The nitrated protein, purified as described previously (3). Trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone was purchased from Worthington and tetraniromethane from Aldrich. NADH, NADP(H), $\alpha$-ketoglutarate, and iodoacetamide were products of Calbiochem. 2'-Monophospho-dinosine-5'-diphosphoribose (the analog of NADP lacking the nicotinamidic ring) was the generous gift of Dr. Nathan O. 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(NO$_3$)$_2$ (as a 0.084 M solution in absolute ethanol) to a solution of the enzyme (1.0 mg/ml) at 30°C 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(NO$_3$)$_2$, followed by adjustment of the pH to 4.5 and dialysis against 5% acetic acid prior to amino acid analysis.

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 tetraniromethane from Aldrich. NADH, NADP(H), $\alpha$-ketoglutarate, and iodoacetamide were products of Calbiochem. 2'-Monophospho-dinosine-5'-diphosphoribose (the analog of NADP lacking the nicotinamidic ring) was the generous gift of Dr. Nathan O. 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.

The abbreviations used are: C(NO$_3$)$_2$, tetranitromethane; A(2'-P)RPPR, 2'-monophospho-dinosine-5'-diphosphoribose; R$_{nap}$, mobility relative to aspartic acid in paper electrophoresis at pH 1.0.
reduction, the cysteiny1 residues were carboxymethylated at 37°
by treatment with a 1.2-fold molar excess, relative to reductant, of
iodacetamide. After 25 min, 20 μl of mercaptoethanol were added and
the alkylated protein was dialyzed against 50% acetic acid.

Molecular Weight Determinations—Polyacrylamide gel electropho-
resis in the presence of sodium dodecyl sulfate was performed as
described by Weber and Osborn (5). In some experiments, the nitrated
protein was not subjected to reduction prior to electrophoresis.
The molecular weight of the subunit of the nitrated enzyme was estimated
by comparing its mobility in such gels to an uninfected control and to
the following standard proteins: the subunit of bovine glutamate
dehydrogenase (55,300), ovalbumin (43,300), diisopropylphosphoro-
dihydidephosphatase (23,300), ribonuclease A (11,700), and horse
heart cytchrome c (11,700).

Isolation of Labeled Peptide—The nitrated, carboxymethylated
protein, which was insoluble in the absence of denaturants,
was solubilized by maleylation (6) and hydrolyzed with 4% by weight of
trypsin at 40° in 0.1 M NH₄HCO₃ buffer at pH 8.1. After 3 hours, the
preparation was adjusted to pH 3.0 and the peptide mixture was
demaleylaied by incubation at 40° for 36 hours. The resulting
susension of peptides was again treated with trypsin (2.5%) as
described above. The small amount of insoluble material remaining at
the end of the digestion was collected by centrifugation. 3-Nitrotyro-
sine was presumed to be absent from the colorless precipitate which
was discarded.

Fractionation of the trypic digest was performed by gel filtration on
a column (1.8 x 150 cm) of Sephadex G-25 (fine) with 0.05
M NH₄HCO₃ as the solvent. Peptides were detected by their absorbance
at 280 nm and peptides containing 3-nitrotyrosine residues by virtue of
their absorbance at 428 nm.

The 428-nm absorbing fractions were pooled, repeatedly lyophilized
to remove the volatile salt, and subjected to preparative paper
electrophoresis at pH 1.9. Only a single peptide spot in the mixture
turned yellow when sprayed with 10% ammonia solution, indicating
the presence of 3-nitrotyrosine.

Amino acid analysis of the eluted material indicated that the
nitrotyrosine-containing fraction was not completely homogeneous and
containing 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°
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
value in this system (9) and estimated with the ninhydrin color value
for tyrosine (8).

RESULTS

Reaction with Tetranitromethane—The course of the reaction
of C(NO)ₓ₄ (8.4 x 10⁻⁴ M) with the enzyme (1.1 mg/ml) at
30° 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 to Fig. 1,
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 nitrated enzyme, which was subse-
sequently reduced and carboxymethylated, indicated the
presence of 1.1 residues of 3-nitrotyrosine per subunit and no
loss of histidine or methionine, residues that might have
reacted with C(NO)ₓ₄. In addition, 1.4 residues of cysteic acid
were found with a corresponding decrease to 4.3 residues of
S-carboxymethylcysteine per subunit.

Protection of Enzyme Activity—Treatment of the enzyme
with C(NO)ₓ₄ in the presence of 10 mM α-ketoglutarate failed
to protect the enzyme activity (Fig. 2). However, when the
reaction with C(NO)ₓ₄ 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

C(NO)ₓ₄ 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)ₓ₄, indicating that protection by NADP or NADPH was
specific for the coenzyme. Low levels of NADP (0.1 mM)
afforded partial protection against inactivation by C(NO)ₓ₄
(data not shown).

In order to investigate which portion of the coenzyme
molecule might be responsible for the protective effect, the
nitrination 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 preven
inactivation of the enzyme by C(NO)ₓ₄, 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)ₓ₄ in the presence of 10 mM or 0.1 mM nicotinamide

![Fig. 1. Time course of the reaction of Neurospora glutamate
dehydrogenase (1 mg/ml) with tetranitromethane (8.4 x 10⁻⁴ M) at pH
8.0 and 30°. Inset, semilogarithmic plot of the course of the reaction.
](http://www.jbc.org/)

![Fig. 2. Effect of various ligands on the rate of inactivation of the
enzyme. All ligands were tested at a concentration of 10 mM; control,
no additions; Δ, α-ketoglutarate; NAD(H); O, 2'-monophospho-
dehydrieosine-S'-diphosphoribose; N-methylnicotinamide; O,
NADP(H); NN, NMN(H).](http://www.jbc.org/)
mononucleotide (reduced form). As shown in Fig. 2, the presence of 10 mM NMNH completely protected the enzyme activity against C(NO)$_3$. 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)RPBR and NMN are inhibitors of the enzyme. Within experimental error, both substances are competitive with NADP in the oxidation of glutamate. The determined $K_i$ values are $4.30 \times 10^{-4}$ M for A(2'-P)RPBR and $4.60 \times 10^{-3}$ M for NMN, as compared to $K_i = 5.6 \times 10^{-4}$ M for NADP$^+$ (10).

Molecular Weight of Nitrated Glutamate Dehydrogenase

The effect of nitration upon the subunit molecular weight was determined by sodium dodecyl sulfate gel electrophoresis either in the presence or the absence of mercaptoethanol. 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 nmol of enzyme with C(NO)$_3$. followed by carboxamidomethylation, gave a preparation containing 1.1 residues of 3-nitrotyrosine per subunit. After hydrolysis with trypsin, the soluble tryptic peptides were applied to a column (1.8 x 150 cm) of Sephadex G-25. The elution profile is shown in Fig. 4. Only a single peak, Fraction 6, showed significant absorption at 428 nm. Based on the molar extinction coefficient, $\varepsilon = 4100$, for 3-nitrotyrosine, the material under Peak 6 accounted for 78% of the 3-nitrotyrosine (1.56 pmol) in the protein preparation.

Fraction 6 was further purified by preparative electrophoresis at pH 1.9. After spraying with ninhydrin, a test strip showed that at least five components were present; however, only one ($R_{exp} = 0.75$) turned yellow when sprayed with 10% NH$_3$.OH. 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, thus indicating that no cross linking of the subunits had occurred.

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. Peptide CN-1 having $R_{exp} = 1.0$ and Peptide CN-2 having $R_{exp} = 1.9$. The compositions (Table I) were those representing residues 160 through 164 and residues 165 through 170, respectively, and provided sufficient information to identify the site of nitration as tyrosine-168.

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(NO)$_3$.8

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**Fig. 3.** Competitive inhibition of glutamate dehydrogenase by nicotinamide mononucleotide or 2' monophosphoadenosine-5' diphosphoribose. Oxidative deamination was measured in the presence of either 2.36 mM NMN (●) or 1.42 mM A(2'-P)RPBR (△) plus coenzyme, or with coenzyme alone (○).

**Fig. 4.** Elution pattern of tryptic peptides derived from nitrated, carboxamidomethylated glutamate dehydrogenase. Chromatography was performed on a column (1.8 x 150 cm) of Sephadex G-25 (fine) with 0.05 M NH$_2$CO$_2$ as solvent. Fractions of 3.4 ml were collected at a flow rate of 34 ml/hour and monitored for absorbance at 280 nm (——) and at 428 nm (---).

**Fig. 5.** Amino acid sequences for the bovine (B) and chicken (C) glutamate dehydrogenases and the Neurospora (N) enzyme in the region including the nitrated tyrosine residue (TYR), number 168. Residues identical in the three enzymes are enclosed in boxes; those differing by single base changes in the amino acid codons are underlined. The tryptic peptide, residues 160 through 170, including the 3-nitrotyrosine was isolated in crude form. After cleavage with CNBr, the two resulting fragments, Peptides CN-1 and CN-2 (Table I) were obtained.
suggestions (11, 12) that residues 219 through 254 of the moiety of the coenzyme. This would be in accord with earlier phoadenosine-5′.diphosphoribose does not protect indicates inactivation. Furthermore, the finding that 2′.monophosphate dehydrogenases with other dehydrogenases of known specificity of this protection was demonstrated completely prevented by the presence of the coenzyme NADP or by showing that NAD or NADH is without effect on the rate of NADPH. The specificity of this protection was demonstrated.

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 (Neurospora numbering) of these enzymes.

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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