Partial amino-acid sequence of NAD-specific glutamate dehydrogenase of *Neurospora crassa*

(large-subunit enzyme)

BRIAN M. AUSTEN, JOSEPH F. NYC, YAIR DEGANI*, AND EMIL L. SMITH†

Department of Biological Chemistry, UCLA School of Medicine, and Molecular Biology Institute, University of California, Los Angeles, Calif. 90024

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ABSTRACT Parts of the primary structure of the NAD-specific glutamate dehydrogenase [L-glutamate:NAD oxidoreductase (deaminating), EC 1.4.1.2] from *Neurospora crassa* are presented. Segments of the sequence representing 886 unique amino-acid residues have been determined; the largest contains 267 residues. There are only short regions of possible homology between this enzyme and the glutamate dehydrogenases of bovine liver or the NADP-specific enzyme of *Neurospora*. The large size of the subunit (116,000 molecular weight) of the NAD-specific glutamate dehydrogenase is unusual when compared to other known dehydrogenases.

The properties of glutamate dehydrogenases (GDHs) differ in ways that depend both on the biological source and on the presumptive metabolic role of the enzyme (1). The vertebrate enzymes function with either NAD or NADP, and are subject to allosteric regulation by purine nucleoside di- and triphosphates. Enzymes of plant or microbial origin are usually specific for one of the coenzymes and are not allosterically regulated. Two GDHs, one requiring NAD, and the other NADP, are found in *Neurospora crassa* (2). The levels of both enzymes are regulated by glutamate (or other sources of nitrogen such as urea, ammonia, or alanine), which represses the synthesis of the NADP-GDH, but derepresses synthesis of the NAD-GDH (3, 4). During growth of the mold there is an inverse relationship between the levels of the two enzymes. The regulatory effects of glutamate suggest that NAD-GDH primarily plays an energy-supplying catabolic role, and NADP-GDH, a biosynthetic one.

The NADP-GDH, like the GDHs of vertebrates, is composed of six identical subunits (5). Its polypeptide chain comprises 452 amino-acid residues, 48 less than the subunit of bovine GDH, and there is considerable homology in the sequences of the NH₂-terminal halves of the two enzymes (6). The purpose of this report is to present the current status of our studies on the structure of the NAD-specific GDH [L-glutamate:NAD oxidoreductase (deaminating), EC 1.4.1.2] of *Neurospora*.

MATERIALS AND METHODS

The purification of NAD-GDH has already been described (7). Carboxypeptidase Y (8) was a gift from Dr. R. Hayashi, pyrrolidone carboxylic acid (PCA)-peptidase (9) from Dr. R. E. Fellows, and staphylococcal protease (10) from Dr. G. R. Drapeau. Other materials were obtained from commercial suppliers.

Abbreviations: GDH, glutamate dehydrogenase; NAD-GDH, NAD-specific glutamate dehydrogenase; NADP-GDH, NADP-specific glutamate dehydrogenase; PCA, L-2-pyrrolidone-5-carboxylic acid.

* Present address: Department of Organic Chemistry, The Weizmann Institute of Science, Rehovot, Israel.

† To whom reprint requests should be addressed.

Fragments were obtained from the denatured, S-carboxymethylated protein by cleavage with cyanogen bromide[‡] or trypsin[§], and by tryptic hydrolysis of the maleylated protein[¶]. Peptides were fractionated on columns, first by chromatography on Sephadex in 30% acetic acid or 6 M guanidinium chloride. Smaller peptides were then purified by elution from Dowex 1 with pH gradients, or from SP-Sephadex by salt gradients in 30% acetic acid, followed by electrophoresis or chromatography on paper. Larger peptides were purified further on columns by elution in 6–8 M urea from CM-cellulose or DEAE-cellulose with salt gradients at constant pH. Purity of large peptides was established by electrophoresis at pH 2.7 on polyacrylamide containing 10–15% monomer, and either 2.5 M or 6.25 M urea. Secondary hydrolysis of large peptides was performed with trypsin, chymotrypsin, staphylococcal protease, thermolysin, or papain. Peptides were degraded by the manual Edman method (11) or by the Beckman sequenator. Sequences close to the COOH-terminus of peptides were determined by hydrolysis with carboxypeptidases A, B, or Y. Leucine aminopeptidase, aminopeptidase M, and tripeptidase (12) were also used. Amides were identified as the phenylthiohydantoin, by analysis of the products of exopeptidase action, or by electrophoresis at pH 6.5. Other methods have been previously described from this laboratory (13).

RESULTS

Earlier physical studies indicated that NAD-GDH comprised four polypeptide chains each containing more than 1000 residues. We have presently placed in unique sequence fragments comprising 886 residues. The longest three sequences include 267, 134, and 101 residues; a total of 674 residues in unique sequences is shown in Figs. 1 and 2. These include only those fragments containing at least 23 residues with one exception (see below).

Certain difficulties were experienced in resolving the peptides. At least six Asp-Pro bonds, which are labile under the acidic conditions (14) used during the isolation of some of the CNBr peptides, occur in the protein. The complexity of this digest was also increased by the presence of at least two Met-Ser and three Met-Thr bonds, which are not cleaved quantitatively by cyanogen bromide (15).

Hydrolysis of the maleylated protein by trypsin proceeded rapidly, as measured by the uptake of base required to keep pH constant. Digestion was stopped after 40 min, but several resistant bonds at arginine residues, which were not

‡ B. M. Austen and E. L. Smith, in preparation.

§ Y. Degani, J. F. NYC, and E. L. Smith, in preparation.

¶ B. M. Austen, J. F. NYC, and E. L. Smith, in preparation.

FIG. 1. The amino-acid sequences of eight segments of *Neurospora* NAD-GDH. Only peptides providing overlaps are shown. The single cysteine residue of the native enzyme that reacts with sulfhydryl reagents is at position 69 of Fragment I (11). Peptides are designated as either CN (cyanogen bromide), T (tryptic), or TM (tryptic peptides from the maleylated protein). Asp-Pro bonds that were found to have undergone partial cleavage are marked with double vertical lines.

hydrolyzed completely (e.g., Fragment IV in Fig. 1), resulted in a larger number of peptides to be purified.

The NH₂-terminus of the CNBr peptide comprising the first 50 residues of Fragment I contained PCA; this was confirmed by its liberation by PCA-peptidase from a tripeptide obtained by a papain digest of a chymotryptic peptide derived from the CNBr peptide. The sequence around the single reactive cysteine residue, modification of which by bulky or charged substituents results in reduced binding of both coenzyme and substrate (11), has been corrected and extended in Fragment I (Fig. 1).

Only three residues were identified by the automated Edman procedure at the NH₂-terminus of Fragment III, a CNBr peptide of 134 residues; the low yield at the third step may have been caused by rearrangement of the asparaginyl residue to the imide or β -form (16). The sequence was solved by isolation of staphylococcal protease peptides and tryptic peptides from the maleylated Fragment III.

Fragment VIII represents the seven COOH-terminal resi-

dues of the protein, since both the CNBr peptide and the overlapping tryptic peptide contain COOH-terminal glutamic acid.

DISCUSSION

At the present time, most enzymes, whether consisting of a single chain or multiple subunits, are of relatively modest size, the overwhelming majority containing polypeptide chains of less than 500–600 residues. Most GDHs are hexameric proteins and the chains of known sequence contain from 452 residues (*Neurospora* NAD-GDH) to 500–503 residues (bovine and chicken) (1). Earlier studies indicating a tetrameric structure for the NAD-GDH of *Neurospora* with each chain comprising more than 1000 residues was, therefore, somewhat surprising. In view of the disparity between the two kinds of GDHs it was desirable to establish the true size of the NAD-GDH subunit and, if indeed, there are such large differences among the GDHs of different origin and

FIG. 2. The largest fragment of *Neurospora* NAD-GDH so far sequenced, containing 267 amino-acid residues. Only two of the peptides derived by further digestion of CNBr peptides are shown. Other designations are given in the legend to Fig. 1.

coenzyme specificity, what structural and functional relations exist among them.

As listed in Table 1, the composition of the presently available peptides, which have been placed in unique sequences, accounts for 886 residues (not all of which are shown in Figs. 1 and 2) out of the total of 1030, as calculated from the amino-acid analysis based on a subunit molecular weight of 116,000 (7). These data demonstrate that the completed sequence will have a molecular weight close to that measured by sedimentation-equilibrium of the subunits. Further, the subunit cannot contain any significant amount of repetitive sequences.

As yet, the blocked NH_2 -terminal sequence (7) of the enzyme has not been identified and the seven residue COOH-terminal sequence (Fragment VIII) has not been extended. Since the positioning of the fragments shown in Figs. 1 and 2 is unknown, it is difficult to compare on an absolute basis the

presently known segments of the NAD-GDH with those of other GDHs. It is already evident, however, that there is no close homology between the fragments shown in Figs. 1 and 2 and other GDHs. Nevertheless, certain short regions of the NAD-GDH do appear to be similar in sequence to those of other GDHs; three of these are shown in Fig. 3.

The first sequence, α , containing a lysyl residue (marked with a plus sign) that is essential for the activity of the bovine and *Neurospora* NADP-enzyme (1) resembles a sequence in Fragment IX of NAD-GDH. Sequence β of the *Neurospora* NADP-GDH contains a tyrosyl residue, nitration of which inactivates the enzyme (17); this sequence is similar to one in the NAD-GDH. The significance of this similarity does not prove homology of function, although it is known that tetranitromethane rapidly destroys the activity of the tetrathionate-treated NAD-GDH.[†] The γ sequence includes a five-residue sequence that is identical in bovine

FIG. 3. Similarities in sequence between certain parts of NAD-GDH and sequences of bovine and *Neurospora* NADP-GDHs. In the α sequence, the residues marked + are reactive with pyridoxal phosphate; in the β -sequence, the residue marked * reacts with tetranitromethane. The residues marked with boxes are identical, and those underlined are related by single base changes in their codons. B, bovine GDH; NADP, *Neurospora* NADP-GDH; NAD, *Neurospora* NAD-GDH.

Table 1. Comparison of composition of NAD-GDH and number of unique residues so far found in sequenced peptides

	Composition (7)	Sequence
Tryptophan	7	6
Lysine	65	66
Histidine	25	17
Arginine	63	61
Aspartic acid	114	104
Threonine	48	43
Serine	74	66
Glutamic acid	103	93
Proline	49	40
Glycine	66	57
Alanine	71	56
Cysteine	9	6
Valine	69	53
Methionine	20	19
Isoleucine	67	53
Leucine	92	81
Tyrosine	41	33
Phenylalanine	47	33
Total residues	1030	886

and NAD-GDH, the longest identical sequence yet found.

The lack of extensive sequence homology with other GDHs and the large size of the subunit show that the *Neurospora* NAD-GDH is a most unusual enzyme. Isolation of the remaining large peptides required to complete the sequence and the overlaps of NAD-GDH should permit a better insight into the functional domains of this unusually large enzyme and its possible relationships to other dehydrogenases.

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