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Soluble reduced nicotinamide adenine dinucleotide oxidase from *Bacillus cereus* T spores and vegetative cells. II. Properties

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Two soluble reduced nicotinamide adenine dinucleotide (NADH₂) oxidases purified from extracts of *Bacillus cereus* T spores were compared with vegetative cell soluble NADH₂ oxidase. The minor spore component and vegetative cell soluble NADH₂ oxidase reacted equally well with riboflavin or flavin mononucleotide (FMN), were inhibited by 15 mM dipicolinic acid (DPA), and possessed similar thermal inactivation characteristics at 80 °C. Activity of the major spore component was stimulated by a factor of 3.6 when riboflavin replaced FMN as the coenzyme. The major spore component was not inhibited by DPA and resisted heat treatments which inactivated vegetative cell soluble NADH₂ oxidase. These observations indicate that the minor spore component and vegetative cell soluble NADH₂ oxidase are identical while the major spore component is a distinct protein.

Introduction

Spores and vegetative cells of *Bacillus cereus* T contain a soluble reduced nicotinamide adenine dinucleotide (NADH₂) oxidase which reportedly is a flavoprotein dependent upon flavin mononucleotide (FMN) for activity (3). The soluble NADH₂ oxidase activity in extracts from *B. cereus* T spores separates into two distinct fractions during preparative acrylamide gel electrophoresis, whereas that of vegetative cell extracts moves as a single component (1). The faster moving spore component constitutes 30% of recovered activity and is identical with vegetative cell soluble NADH₂ oxidase in electrophoretic mobility on acrylamide gel. The major spore component moves more slowly than its vegetative cell counterpart during acrylamide gel electrophoresis at pH 8.3. The purpose of the present investigation was to compare some of the physical and chemical properties of the soluble NADH₂ oxidases of *B. cereus* T spores and vegetative cells.

Materials and Methods³

Organisms and Cultural Conditions

B. cereus T QMB 1590 and *B. megaterium* QMB 1551 were supplied by H. L. Levinson, Pioneering Research Division, U.S. Army Natick Laboratories, Natick,

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Massachusetts. *B. cereus* NRS 804 and *B. subtilis* 15 u were obtained from the Dairy Products Laboratory culture collection. The conditions of growth, procedures for harvesting, washing, and breakage of vegetative cells and spores, and purification of soluble NADH₂ oxidases have been described previously (1).

Assay Procedures

Soluble NADH₂ oxidase activity and protein concentrations were assayed as described by Ashton and Blankenship (1). Where indicated, the reaction mixture contained 0.1 μmole of FMN, flavin adenine dinucleotide (FAD), or riboflavin. A unit of enzyme activity, which corresponds to the oxidation of 0.16 μmoles of NADH₂, was defined as the amount of protein causing an absorbance change of 0.001 per minute. Specific activity was the number of units per milligram of protein.

Determination of Coenzyme Requirement

The presence of riboflavin in spore extracts was confirmed by means of a thin-layer chromatography (t.l.c.) procedure. Crude soluble extracts of spores and vegetative cells each containing 10 mg protein per milliliter were spotted in 10- and 25-μl quantities on plates of silica gel H (Brinkmann Instruments, Inc., Westbury, N.Y.). Plates were developed in acetone : glacial acetic acid : methanol : benzene (5:5:20:70) (5) and visualized with ultraviolet light.

Cofactor requirement of the soluble NADH₂ oxidases from spores and vegetative cells of *B. cereus* T was confirmed after removing flavin from protein by acid ammonium sulfate treatment (14).

Thermal Inactivation

Reaction mixtures containing 50 μmoles of tris(hydroxymethyl)aminomethane (Tris) chloride, pH 7.8, and purified enzyme in a volume of 1.2 ml were heated in a water bath at 80 °C for the desired length of time. After cooling in an ice bath, FMN and substrate were added and residual NADH₂ oxidase activity determined. Protein concentrations used in thermal inactivation studies were equivalent for both spore and vegetative cell enzymes.

TABLE I
Flavin coenzyme stimulation of NADH₂ oxidases in crude soluble extracts of
Bacillus cereus T spores and vegetative cells

Source of extract	Specific activity*	Stimulation factor		Activity ratio, riboflavin/FMN
		FMN	Riboflavin	
Vegetative cell	50.0	3.0	3.3	1.1
Spore	20.0	5.0	17.0	3.4

*No cofactor added.

Results

Cofactor Requirement of Soluble NADH₂ Oxidase

NADH₂ oxidase activity in crude soluble extracts of *B. cereus* T spores and vegetative cells was stimulated by the addition of 0.1 μmole of FMN or riboflavin to the reaction mixture. With vegetative cell extracts, the stimulation was about threefold for each of the cofactors (Table I). With spore extracts, addition of FMN increased NADH₂ oxidase activity fivefold whereas riboflavin caused a 17-fold stimulation, representing a 3.4-fold improvement over FMN activation. Similar responses to riboflavin were observed with soluble extracts prepared from spores of other species of *Bacillus*. Soluble NADH₂ oxidase activity in crude extracts of *B. megaterium* 1551 was increased by a factor of 3.3 when riboflavin was added in place of FMN as the coenzyme. The corresponding factors for *B. cereus* 804 and *B. subtilis* 15 u spore extracts were 3.3 and 2.6, respectively. In each case, NADH₂ oxidase in soluble extracts from the vegetative cell counterparts reacted equally well with either FMN or riboflavin. The same riboflavin/FMN activity ratios were observed in vegetative cell and spore extracts from which flavin had been removed by acid ammonium sulfate treatment.

When FAD was used as the coenzyme, soluble NADH₂ oxidase activities of both vegetative cell and spore extracts were 30% lower than with FMN. This was in agreement with the observations of others (4). The observed stimulations were not improved by the addition of greater than 0.1 μmole of flavin coenzyme.

The presence of riboflavin in spore extracts was confirmed by a t.l.c. procedure. Riboflavin was detectable in 10 μl of a spore extract containing 10 mg protein per milliliter but was not detected in 25 μl of vegetative cell extracts containing the same protein concentration.

As previously described, the soluble NADH₂ oxidases of *B. cereus* T spores and vegetative cells were purified by preparative acrylamide gel electrophoresis (1). Two distinct components with NADH₂ oxidase activity were found in spore extracts while vegetative cell extracts contained only one. After purification on acrylamide gel, the enzymes required the addition of a flavin coenzyme for activity. The purified vegetative cell enzyme and spore peak one reacted equally well with either FMN or riboflavin (Table II). Enzyme in spore peak two oxidized NADH₂ 3.6 times faster when FMN was replaced by an equal concentration of riboflavin in the reaction mixture. Before pooling the fractions of spore peak two, one fraction was observed to have a maximum activity ratio (riboflavin/FMN) of 5.3.

Inhibition and Activation

Dipicolinic acid (DPA) has been reported to replace FMN partially as the coenzyme of *B. cereus* T soluble NADH₂ oxidase. In addition, DPA decreased the rate of FMN-mediated activity, suggesting a competition for the enzyme (4).

In the present study, DPA stimulation of enzyme from which flavin had been removed by acid ammonium sulfate treatment could not be demonstrated either in crude extracts or purified preparations. DPA inhibition of soluble NADH₂ oxidases was observed in crude extracts of both

TABLE II
Coenzyme requirement of purified soluble NADH₂ oxidases from vegetative cells and spores of
Bacillus cereus T

Source of enzyme	% of total activity	Activity ratio, riboflavin/FMN
Vegetative cell	100	1.1
Spore peak one	30	1.1
Spore peak two	70	3.6

spores and vegetative cells. After purification of the enzymes, the effect of DPA on riboflavin- or FMN-mediated activity was reexamined (Table III). Activities of both the vegetative cell enzyme and spore component one were inhibited about 50% by 15 mM DPA. Riboflavin-

or FMN-mediated activity of spore component two was affected only slightly by DPA.

The addition of 15 mM calcium to the reaction mixture stimulated FMN-mediated activities of vegetative cell NADH₂ oxidase and spore component one by a factor of 1.4. The corresponding factor for spore component two was 1.9. Calcium was also effective in relieving DPA inhibition when added in an equimolar concentration. The stimulatory effect of several other divalent cations was tested. Of these, strontium, magnesium, and manganese functioned in the same manner as calcium. Divalent cations did not stimulate riboflavin-mediated NADH₂ oxidase activity.

TABLE III

 Comparison of *Bacillus cereus* T vegetative cell and spore purified soluble NADH₂ oxidases

Cofactor	Activity*	Ratio to FMN stimulation
Vegetative cell		
None	0.0	—
FMN	2.0	—
DPA (15 mM)	0.0	—
FMN + DPA	1.1	0.55
FMN + Ca (15 mM)	2.8	1.40
FMN + Ca + DPA	2.1	1.05
Spore peak one		
None	0.0	—
FMN	5.0	—
DPA	0.0	—
FMN + DPA	3.0	0.60
FMN + Ca	7.0	1.40
FMN + Ca + DPA	4.7	0.93
Spore peak two		
None	0.0	—
FMN	5.0	—
DPA	0.0	—
FMN + DPA	4.5	0.9
FMN + Ca	9.5	1.9
FMN + Ca + DPA	4.5	0.9

* μ moles NADH₂ oxidized per minute per milligram protein.

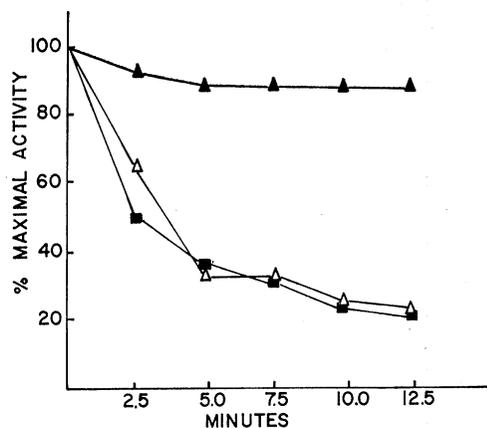


FIG. 1. Heat inactivation at 80°C of soluble NADH₂ oxidases derived from spores (Δ electrophoresis peak 1; ● peak 2) and vegetative cells (■) of *Bacillus cereus* T in 0.04 M Tris-chloride, pH 7.8.

Thermal Inactivation

The thermal inactivation characteristics of purified vegetative cell soluble NADH₂ oxidase and the two spore components were studied (Fig. 1). At 80°C in 0.04 M Tris-chloride, pH 7.8, the major spore component lost 10% of its activity in the first 5 minutes of heating. Further losses of activity were not evident. The vegetative cell enzyme and spore component one, on the other hand, lost 70% of the original activity during the first 5 minutes at 80°C. A further gradual loss was observed upon more extended heating of these two enzymes.

Discussion

When confronted with conditions which limit or prevent growth, many microorganisms undergo intracellular proteolysis (8, 15, 16). Such is the case immediately before and during spore formation in *Bacillus* species. As a result, much of the vegetative cell protein is degraded before the appearance of refractile spores. Spore protein, therefore, is regarded as being largely newly synthesized (2, 9, 11, 16).

Numerous comparisons between spore and vegetative cell enzymes have been made to determine whether or not newly synthesized spore proteins differ from their vegetative cell counterparts. Many have proved to be identical on the basis of physical and functional properties (7) while others are reported to possess distinct kinetic, immunologic, and heat resistance characteristics (6, 10, 12).

Ashton and Blankenship (1) found that the soluble NADH₂ oxidases of *B. cereus* T spores

and vegetative cells differed in electrophoretic mobility on acrylamide gel. As much as 30% of the NADH₂ oxidase activity in soluble extracts of spores was due to the presence of an enzyme with electrophoretic mobility on acrylamide gel identical with that of vegetative cell soluble NADH₂ oxidase. Spore extracts contained, in addition, a unique soluble NADH₂ oxidase. In the present study, some of the properties of the three enzymes were investigated. In every case, the minor spore component proved to be identical with vegetative cell soluble NADH₂ oxidase, whereas the major spore component was a distinct protein. These findings confirmed observations made during the acrylamide gel electrophoresis step of purification.

The most significant difference between the soluble NADH₂ oxidases from spores and vegetative cells was that of coenzyme requirement. While the vegetative cell enzyme functioned equally well with either FMN or riboflavin, spore enzyme of electrophoretic peak two was almost four times as active with riboflavin. The occurrence of riboflavin in soluble extracts of spores was confirmed by t.l.c., suggesting that riboflavin was indeed the coenzyme used by soluble NADH₂ oxidase of spores. Riboflavin could not be detected in vegetative cell extracts.

The number of known flavoproteins using FMN or FAD as the coenzyme is large and such enzymes are widely distributed in nature. Warburg's "old yellow enzyme", which requires FMN for activity, and D-amino acid oxidase, which uses FAD, are classic examples. Studies on the interactions between flavin phosphates and several FMN-dependent enzymes revealed that the phosphorylated derivatives bind and function with apoenzymes much more effectively than do the corresponding free flavins (13). This evidence implicates the anionic phosphate group of FMN as a common site of attachment to the apoenzymes.

In the present study, a further indication that FMN and the major spore component react with difficulty was the twofold stimulation of FMN-mediated activity by certain divalent cations. Perhaps the approach of FMN to the coenzyme site is hindered by the negatively charged phosphate group. Divalent cations may facilitate this approach by cancelling the negative charge. Riboflavin, which is without

the phosphate group altogether, reacts even more readily with the apoenzyme. Furthermore, the addition of calcium has no stimulatory effect upon riboflavin-mediated NADH₂ oxidase activity of the major spore component.

In addition to possessing an altered coenzyme requirement, the major soluble NADH₂ oxidase of spores possesses a greater resistance than its vegetative cell counterpart to inhibition by DPA. This unique enzyme may provide an important protective mechanism for the spore during the final stages of sporulation and early stages of germination since DPA comprises 10 to 15% of the dry weight of spores but is completely absent in vegetative cells. The small amount of DPA inhibition (10%) of the major spore component is probably due to contamination of this fraction by the minor spore component. The same contamination is thought to account for the rapid initial loss of 10% of the original activity in spore component two during heating at 80 °C.

Thus, the soluble NADH₂ oxidase unique to spores is significantly different from its vegetative cell counterpart, resulting in a definite preference for riboflavin over FMN as the coenzyme. In addition, the unique spore enzyme possesses a degree of resistance to DPA inhibition and thermal inactivation which is not apparent with the vegetative cell soluble NADH₂ oxidase. These properties are important to the bacterial spore in view of its unique composition and characteristics. We are currently investigating the possibility that the major spore soluble NADH₂ oxidase of *B. cereus* T is generated from its vegetative cell counterpart by limited proteolysis as has been suggested in the case of fructose-1,6-diphosphate aldolase (12).

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