BBA 43272

## Terminal oxidations in Bacillus Brevis ATCC 10068. I. The measurement of the NADH oxidase activity of Bacillus Brevis ATCC 10068

During an investigation of the oxidative metabolism of the tyrothricin producing organism *Bacillus brevis* ATCC 10068 (ref. 1) we observed the presence of an active NADH oxidase system. Measurement of the oxidase activity was, in some instances complicated by the presence of an interacting system. We wish to report on the characteristics of this system and its effect on measurements of NADH oxidase activity.

The organism was grown aerobically at 37° under shake conditions in a glycerol–asparagine medium supplemented with salts[1]. The logarithmic phase of growth typically commenced after a lag period of 18 h and was sustained for 6–8 h.

Whole cell activity was measured with cells harvested in the logarithmic phase of growth, washed twice in the growing medium *minus* substrates and resuspended in the same medium. The cells were resuspended in 0.1 M Tris–HCl buffer (pH 7.4) when they were to be subsequently fractionated. Membrane and supernatant fractions were prepared by passing whole cells 3 times through a modified French pressure cell[2] at 16000 lb/inch$^2$ at 0°. The cell debris das removed by centrifugation at 15000 $\times g$ for 30 min. Further centrifugation at 105000 $\times g$ for 90 min yielded a red particulate fraction of membrane material and a yellow supernatant fraction. Measurement of NADH oxidase activity was made polarographically by means of a Clark type oxygen electrode[3] following oxygen consumption as NADH was oxidised, or spectrophotometrically by recording the fall in absorbance at 340 nm on addition of NADH.

(*a*) *NADH oxidase activity of whole cells.* Table I shows that the NADH oxidase activity of washed whole cells is low. This low value is most readily ascribed to the impermeability of the bacterial cell membrane to NADH since on incubation with lysozyme there is a considerable increase in the NADH oxidase activity. Lysozyme effectively abolishes the endogenous rate of oxygen consumption normally observed in washed cells. The abolition of endogenous respiration on incubation with lysozyme has also been observed with other organisms susceptible to this enzyme[4].

(*b*) *NADH oxidase activities of subcellular fractions.* The bulk of the NADH oxidase activity is located in the 105 P fraction and relativley lower specific activity was associated with the 105000 $\times g$ supernatant fraction (Table I). Not only was the apparent activity lower in the soluble fractions but also the amount of NADH oxidised accounted for only about 15% of the added NADH incubated with the 150000 $\times g$ supernatant fraction and 33% of that incubated with the 15000 $\times g$ supernatant fraction. All of the added NADH was oxidised by the 105000 $\times g$ particulate fraction fraction. The addition of another aliquot of NADH to the 15000 $\times g$ supernatant or 15000 $\times g$ supernatant fraction initiated further oxidation but this again was only a fraction (33% and 15%, respectively) of the total NADH added. The oxidation of NADPH, however, which was also catalysed by the 105000 $\times g$ particulate fraction and 15000 $\times g$ supernatant fraction fractions proceeded until all the added NADPH was oxidised (calculated from the fall in

TABLE I

OXIDASE ACTIVITIES OF WHOLE CELLS AND SUBCELLULAR FRANCTIONS OF *B. brevis*

The various fractions were prepared as described in the text. Assay system (spectrophotometric) contained Tris–HCl buffer (pH 7.4, 300 μmoles; NADH or NADPH, 0.33 μmole; enzyme protein, 0.2–5.0 mg; total volume 3.0 ml in a 1-cm quartz cuvette. The amount of added substrate oxidised was calculated from the observed $\Delta A_{340\ nm}$ compared to the theoretical $\Delta A_{340\ nm}$ value for the complete oxidation of the nucleotide compound. Assay system (polarographic) was as above except that 1.33 μmoles of NADH or NADPH was used as substrate. Assay system for whole cells (polarographic) contained culture medium *minus* substrates, 3.0 ml; NADH or NADPH, 1.33 μmoles; whole cells protein, 0.5–3.0 mg. All measurements were made at 25°.

| *Preparation* | *Additions* | *Oxidation rate (μ atoms O per min per mg protein)* | | | *% of added substrate oxidised* | |
|---|---|---|---|---|---|---|
| | | *Endogenous* | *NADH oxidase* | *NADPH oxidase* | *NADH* | *NADPH* |
| Whole cells | None | 0.031 | 0.033* | 0.018 | — | — |
| | Lysozyme** | 0.000 | 0.210 | 0.054 | 100 | — |
| 15000 × *g* supernatant fraction | None | 0.000 | 0.080 | 0.023 | 33 | 100 |
| | $NAD^+$(0.5 μmole) | 0.000 | 0.090 | — | 52 | — |
| | $NAD^+$(2.5 μmoles) | 0.000 | 0.110 | — | 70 | — |
| | $F^-$ (30 mM) | 0.000 | 0.123 | — | 100 | — |
| 105000 × *g* supernatant fraction | None | 0.000 | 0.013 | 0.018 | 15 | 100 |
| | $NAD^+$ (0.5 μmole) | 0.000 | 0.015 | — | 23 | — |
| | $NAD^+$(2.5 μmoles) | 0.000 | 0.019 | — | 48 | — |
| | $F^-$ (30 mM) | 0.000 | 0.030 | — | 100 | — |
| 105000 × *g* particulate fraction | None | 0.000 | 0.180 | 0.048 | 100 | 100 |

* Corrected for endogeonus $O_2$ consumption.

** Lysozyme was added (1 mg.) and, immediately the endogenous rate became zero, NADH or NADPH was added.

absorbance at 340 nm on incubation). Addition of a NADH oxidising system (malate dehydrogenase and oxaloacetate) to the incubated system containing NADH and 105000 × *g* supernatant (or 15000 × *g* supernatant fraction) fraction failed to remove the residual absorbance at 340 nm, indicating that the residual absorbance is not due to NADH; this observation was confirmed independently by Dr. B. Hodgson of this Department (personal communication).

$NAD^+$ was found to stimulate both the initial rate and the extent of the reaction of the soluble NADH oxidase (Table I). It appears that $NAD^+$ is acting as a competitive substrate with NADH for what is inferred to be an $NAD^+$–NADH degrading system in the soluble fractions. This was confirmed by the finding that $NAD^+$ was rapidly and completely degraded on incubation with the 105000 × *g* supernatant preparation. This system does not degrade NADPH (as previously indicated by the complete oxidation of NADPH by the NADPH oxidase system of the 105000 × *g* supernatant and 15000 × *g* supernatant fraction fractions) nor does it degrade $NADP^+$ (assayed by an $NADP^+$ specific isocitrate dehydrogenase system[5]).

(*c*) *NAD⁺–NADH degrading system.* The rates of degradation of $NAD^+$ and NADH by the 105000 × *g* supernatant fraction (Fig. 1) were found to be identical when the incubation was carried out under anaerobic conditions (which prevents NADH oxidase activity). On this basis (and since subsequent experiments have

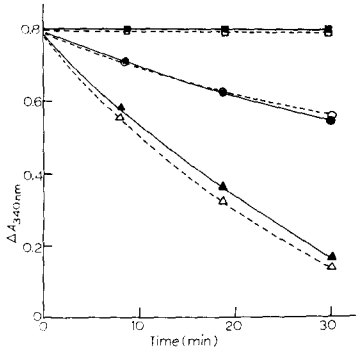


Fig. 1. $F^-$ inhibition of the degradation of $NAD^+$ and NADH by the 105000 × *g* supernatant fraction. $NAD^+$ or NADH was incubated with the 105000 × *g* supernatant fraction 37°. The reaction medium contained: Tris–HCl buffer (pH 7.41, 100 μmoles; $NAD^+$ or NADH, 4 μmoles; 105000 × *g* supernatant fraction enzyme protein, 0.85 mg; KF was added at the concentrations indicated; total volume, 1.0 ml. The NADH incubation was carried out under anaerobic conditions. 0.1-ml aliquots were withdrawn at intervals and the concentration of the nucleotide determined. $NAD^+$ was assayed using a $NAD^+$ specific alcohol dehydrogenase system as follows: Tris–HCl (pH 10.1), 250 μmoles; ethanol, 1.25 mmoles; yeast alcohol dehydrogenase, 50 μg protein; sample volume, 0.1 ml; total volume, 3.0 ml. The increase in absorbance at 340 nm was measured after the addition of sample. NADH was assayed using a NADH specific alcohol dehydrogenase system: phosphate buffer (pH 7.5), 290 μmoles; acetaldehyde, 10 μmoles; yeast alcohol dehydrogenase, 10 μg protein; sample volume, 0.1 ml; total volume, 3.0 ml. The decrease in absorbance at 340 nm was measured after the addition of sample. The solid lines refer to $NAD^+$ degradation; ▲—▲, in the absence of $F^-$; ●—●, with 3 mM $F^-$; ■—■, with 30 mM $F^-$. Broken lines refer to NADH degradation; △—△, in the absence of $F^-$; ○- - -○, with 3 mM $F^-$; □- - -□, with 30 mM $F^-$.

shown $NAD^+$ and NADH degradation reactions to be inhibited to the same extent by a given concentration of fluoride, see Fig. 1) the oxidised and reduced forms of the coenzyme appear to be subject to the same process of degradation. $NAD^+$ was thus used in the subsequent analysis instead of NADH to avoid the unnecessary complication due to NADH oxidase activity. Table II list the results of an examination of the products obtained on incubation of $NAD^+$ with the 105000 × *g* supernatant fraction. The data show that 1 mole of $NAD^+$ gives rise to approx. 1 mole. each of adenosine, NMN and orthophosphate. Analysis of the incubated reaction media for the presence of pyrophosphate, nicotanimide and ADP (by paper chromatography as described under Table II) and for AMP (by AMP deaminase[6]) yielded negative results. The degradation reaction consistent with these observations is.

$$NAD^+ \rightarrow NMN^+ + \text{adenosine} + P_i$$

The $NAD^+$ degradation system was found to be inhibited by $F^-$ (Fig. 1) 30 mM $F^-$ resulted in 100% inhibition of $NAD^+$ degradation. Similarly incubation of 30 mM $F^-$ with the 105000 × *g* supernatant fraction led to higher values for the NADH oxidase activity (Table I). The NADH oxidase activity of a typical 105000 × *g* supernatant fraction increases by about 140% in the presence of 30 mM $F^-$ and

TABLE II

ANALYSIS OF $NAD^+$ DEGRADATION PRODUCTS AFTER INCUBATION OF $NAD^+$ WITH 105000 × *g* SUPERNATANT FRACTION FROM *B. brevis* AT 37°

Incubation system contained: Tris-HCl buffer (pH 7.4), 300 μmoles; $NAD^+$, 4 μmoles; 105000 × *g* supernatant fraction enzyme protein, 0.85 mg; total volume, 3 ml.

| *Product* | *Method of detection and/or estimation* | *Moles of product formed per mole of NAD⁺ degraded* |
|---|---|---|
| Nicotinamide riboside compound | CN complex formation[8] | 1.10 |
| Orthophosphate | *Paper chromatography* *, Fiske–SubbaRow[9] | 1.21 |
| Adenosine | Paper chromatography *, Adenosine deaminase assay[7] | 0.81 |
| NMN | Paper chromatography * | 1.10 ** |

* Paper chromatography was carried out on acid prewashed Whatman No. 1 chromatography paper. Solvent system was either ethanol–0.1 M acetic acid (1:1, v/v) or pyridine–water (2:1, v/v)[10]. Spots were detected under ultraviolet irradiation and by molybdic acid spray[11].

** The pattern of the degradation products obtained indicates the nicotinamide ribosied compound is NMN.

the utilisation of the NADH increases from 15 to 100% (as observed by the fall in absorbance at 340 nm).

Thus the NADH oxidase activity of soluble fractions (15000 × *g* supernatant and 105000 × *g* supernatant) should be assayed in the presence of $F^-$ in order to eliminate interference from the $NAD^+$–NADH degrading system which appears to be a $NAD^+$–NADH specific nucleotide pyrophosphatase.

We are indebted to the Science Research Council for the award of a studentship to one of us (B.S.).

*Department of Biochemistry, University of Manchester Institute of Science and Technology, Manchester (Great Britain)*

B. SEDDON
G. H. FYNN

1 B. MACH, E. REICH AND E. L. TATUM *Proc. Natl. Acad. Sci. U.S.*, 50 (1963) 175.
2 J. R. WALTERS AND D. P. STANLEY, *Appl. Microbiol.*, 16 (1968) 1605.
3 L. C. CLARK, *Trans. Am. Soc. Artificial Internal Organs*, 2(1956) 41.
4 P. L. BROBERG, M. WELSCH AND L. SMITH, *Biochim. biophys. Acta*, 172 (1969) 205.
5 M. M. CIOTTI AND N. O. KAPLAN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods Enzymol*, Vol. 3, Academic Press, New York 1955, p. 890.
6 G. NIKIFORUK AND S. P. COLOWICK, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymol.*, Vol. 2, Academic Press, New York, 1955 p. 469.
7 N. O. KAPLAN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 2, Academic Press, New York, 1955, p. 473.
8 S. P. COLOWICK, N. O. KAPLAN AND M. M. CIOTTI, *J. Biol. Chem.*, 191 (1952) 447.
9 C. H. FISKE Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
10 H. S. BURTON, *Nature*, 173 (1954) 127.
11 S. BURROWS, F. S. M. GRYLLS AND J. S. HARRISON, *Nature*, 170 (1952) 800.

Received June 17th, 1970