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TRANSHYDROGENASE ACTIVITY IN THE MARINE BACTERIUM *BENECKEA NATRIEGENS*

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Summary

The marine bacterium, *Beneckea natriegens*, which has previously been reported not to form transhydrogenase, has been shown to synthesize a soluble energy-independent transhydrogenase (NADPH:$NADP^+$ oxidoreductase, EC 1.6.1.1), though no energy-linked activity could be detected. The transhydrogenase is induced maximally in stationary phase cells and its formation is 70—90% repressed by raising the medium phosphate level from 0.33 to 3.3 mM. The enzyme is inhibited by arsenate, inorganic ortho- and pyrophosphate and by a range of organic phosphate-containing compounds, including 2′-AMP, which is an activator of several bacterial transhydrogenases.

Introduction

Transhydrogenase (NADPH:NAD^+ oxidoreductase, EC 1.6.1.1) catalyses the reaction:

$$NADP^+ + NADH \rightleftharpoons NADPH + NAD^+$$

Transhydrogenases may be energy-independent or energy-linked. Since the redox potential of both nucleotides is about the same, the equilibrium of the energy-independent reaction is poised at about equal concentrations of the oxidised and reduced forms of each nucleotide [1]. When aerobic respiration or ATP hydrolysis is used to drive energy-linked transhydrogenase the equilibrium is strongly in favour of NADPH formation [1]. Mitochondria and some bacteria exhibit both energy-linked and non-energy-linked transhydrogenase activities [1—14], which may [3,15,16] or may not [1,5], be catalysed by the same enzyme. Other bacteria contain only non-energy-linked transhydrogenase [17—21].

The role of transhydrogenase is still not certain. Anabolism requires principally NADPH, whilst catabolism produces mainly NADH. Transhydrogenase

could, therefore, fulfil a vital linking role between catabolism and anabolism [22—24]. *Beneckea natriegens* (formerly *Pseudomonas natriegens* [25,26]) has been reported to not have transhydrogenase and NADPH oxidase activities [23,24]. Catabolism of glucose in *B. natriegens* is 92% by glycolysis and 8% by the hexose monophosphate pathway. Gluconate is 80% degraded by the Entner-Douderoff pathway and 20% by the hexose monophosphate pathway [27]. The hexose monophosphate pathway contains only NADP-linked dehydrogenases. It has been suggested that the utilisation of the hexose monophosphate pathway is primarily for NADPH production, and that this pathway is rate-limited by the supply of $NADP^+$ [23].

These observations are valid only if there is no transhydrogenase and NADPH oxidase present in *B. natriegens*, or if they do not act at a sufficient rate. This paper describes the presence in *B. natriegens* of a non-energy-linked transhydrogenase of unusual properties.

Materials and Methods

B. natriegens strain III was grown to the stationary phase (16 h growth) on a DL-lactate minimal salts medium as described previously but containing only 0.33 mM phosphate [28]. The cells were disrupted by sonication and the cell free extract fractionated into particulate and supernatant fractions by centrifugation at 150 000 $\times g$ [28].

The supernatant fraction contained all the transhydrogenase activity and 3—10% of the NADH oxidase activity. In order to free the transhydrogenase of the contaminating NADH oxidase the enzyme was partially purified. The supernatant fraction was stirred with saturated $(NH_4)_2SO_4$ and the fraction precipitating between 65—85% $(NH_4)_2SO_4$ was collected by centrifugation and dialysed overnight against 50 mM Tris · HCl (pH 7.5) buffer. The dialysate was chromatographed on a DEAE ion-exchange column (DE 52, Whatman) which had been pre-equilibrated with 50 mM Tris · HCl (pH 7.5) buffer. Elution was by a linear gradient of 0.05—0.3 M NaCl in 50 mM Tris · HCl (pH 7.5) buffer. Transhydrogenase eluted as a sharp band at about 0.18 M NaCl. The partially purified enzyme was completely free of NADH oxidase and had been purified 90 fold with a yield of 47%. No further purification was attempted. The eluate was dialysed against 50 mM Tris · HCl (pH 7.5) buffer and stored at —15° C: no loss of activity occurred after 12 weeks storage.

Transhydrogenase was assayed in the direction of NADPH formation in a reaction mixture of 2.9 ml in a 1 cm cuvette containing: 25 μM NADH, 0.3 mg alcohol dehydrogenase, 90 mM ethanol, 50 mM glycine · HCl (pH 9.6) buffer and a suitable concentration of transhydrogenase extract. After 10 min incubation at 35° C the reaction was initiated by addition of 0.1 ml of 15 mM $NADP^+$ and the increase in absorbance measured at 340 nm. In the direction of NADH formation the reaction mixture was 25 μM NAD^+, 0.3 mg alcohol dehydrogenase, 9 mM acetaldehyde and a suitable concentration of transhydrogenase extract in 2.9 ml of 50 mM Tris · maleate (pH 7.0) buffer. After 10 min incubation at 35° C the decrease in absorbance at 340 nm was measured on addition of 0.1 ml of 4.5 mM NADPH.

Yeast alcohol dehydrogenase, glutathione, glutathione reductase, (type II)

NAD^+, NADH, $NADP^+$ and NADPH were obtained from Sigma Chemical Company.

Results and Discussion

Non-energy-linked transhydrogenase

In the presence of an NADH-regenerating system, transhydrogense, partially purified from stationary phase cells of *B. natriegens* grown aerobically on a DL-lactate minimal salts medium, catalyses the reduction of $NADP^+$ as shown by the increase in absorbance at 340 nm (Fig. 1). That the increase in absorbance is due to NADPH formation was confirmed by the rapid decrease in absorbance that occurs on addition of oxidised glutathione and NADPH-specific glutathione reductase (after first neutralising the mixture to a pH where glutathione reductase is optimally active).

There is no activity when the assay is performed in the absence of $NADP^+$, alcohol, alcohol dehydrogenase or transhydrogenase. However, when NAD^+ is omitted 80—100% activity is observed. Dialysis or treatment of the alcohol dehydrogenase and transhydrogenase with Norit (activated charcoal), or passage of the cofactors and enzymes through a small Sephadex column, does not cause any loss of activity when the assay is measured in the absence of added NAD^+. Paper chromatography showed the $NADP^+$ to be free from traces of

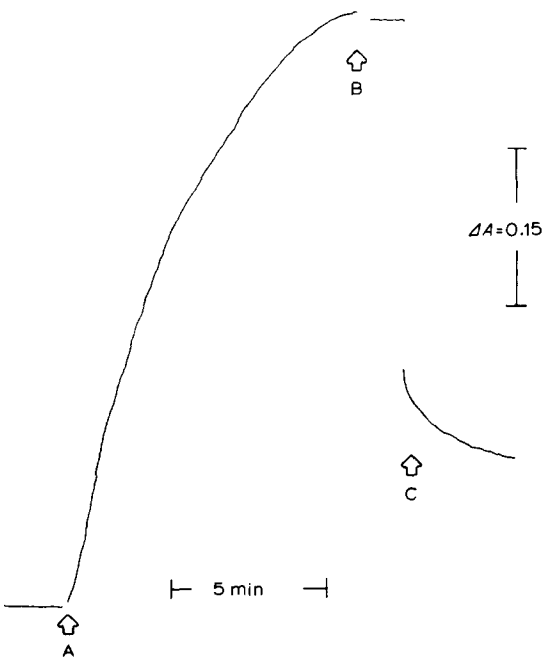

Fig. 1. Time course of $NADP^+$ reduction by NADH. The reaction mixture was 25 μM NAD^+, 0.3 mg alcohol dehydrogenase, 90 mM ethanol and 300 μg transhydrogenase protein in 2.9 ml 50 mM glycine NaOH (pH 9.6) buffer in a 1 cm cuvette. After 10 min incubation at 35°C the reaction was initiated by addition of 0.1 ml 15 mM $NADP^+$ (arrow A). After 10 min reaction time the solution was neutralised to pH 7.2 by addition of 0.1 M HCl (arrow B). At arrow C 0.1 ml 50 mM Tris/maleate (pH 7.2) buffer containing 50 μg glutathione plus 50 μg glutathione reductase was added.

NAD^+. The reaction can also be driven at pH 8—9 by lactate and lactic dehydrogenase (lactic dehydrogenase is essentially inactive at higher pH values) in the absence of added NAD^+. In the crude supernatant fraction, which contains a highly active NAD^+-linked L-malate dehydrogenase, the reaction also proceeds on addition of L-malate in the absence of added NAD^+. The transhydrogenase must therefore contain NAD^+ as a prosthetic group.

pH optima for the enzyme are different in each direction (Fig. 2). In the direction of NADPH formation it is at an unusually high pH (9.6); in the opposite direction it is at pH 7.0. Furthermore the enzyme is much more active in the direction of $NADP^+$ reduction than for NAD^+ reduction. Control experiments show that the NADH or NAD^+ regenerating systems are not rate-limiting.

Transhydrogenases from different bacteria show a remarkable divergence in their properties. The *Pseudomonas aeruginosa* enzyme forms NADPH at a negligible rate unless activated by 2'-AMP, whilst phosphate ions inhibit NADH formation [18]. The enzyme from *Chromatium* is inhibited by 2'AMP and unaffected by phosphate ions [3]. In *Azotobacter vinelandii* transhydrogenase is stimulated by 2'-AMP and inhibited by ADP, ATP and phosphate ions in the direction of NADPH formation, whilst they have little effect on activity in the reverse [19]. Beef-heart transhydrogenase is unaffected by 2'-AMP or phosphate ions [29]. There are wide variations in the sensitivity of various transhydrogenases to Mg^{2+} and in the ability to utilize different substrate analogues, as well as differences in their regulatory properties.

Addition of 5 mM concentrations of a range of compounds containing the phosphate moiety causes 80—100% inhibition of *B. natriegens* transhydrogenase activity in either direction (Table I); in each case 50% inhibition occurs at a 1—2 mM inhibitor concentration. Of particular note is the inhibition by 2'-AMP; lower concentrations of 2'-AMP do not stimulate activity. Arsenate also strongly inhibits activity, whilst adenine, adenosine or Mg^{2+} have little effect.

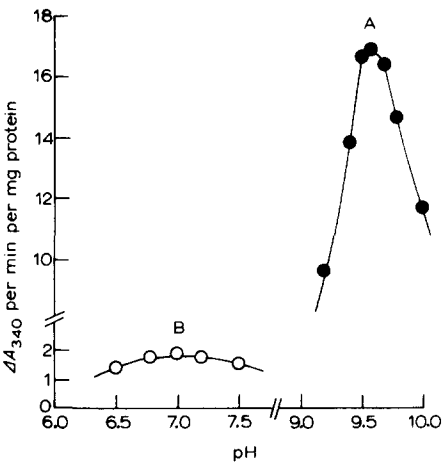

Fig. 2. The pH optima of transhydrogenase in the direction of NADPH formation (curve A, •) and in the direction of NADH formation (curve B, ○).

TABLE I

THE EFFECT OF VARIOUS COMPOUNDS ON THE ACTIVITY OF PARTIALLY PURIFIED TRANSHYDROGENASE

All additives were adjusted to the correct pH (9.6 or 7.0) before addition to the assay mixture.

Additions (5 mM)	% Activity	
	Direction of NADPH formation	Direction of NADH formation
None	100	100
Orthophosphate	0	0
Pyrophosphate	9	n.d. *
Arsenate	0	0
5′-AMP	8	0
2′-AMP	12	0
ADP	6	0
ATP	19	0
Phospho*enol*pyruvate	13	n.d.
TTP (2 mM)	29	n.d.
GTP	11	n.d.
CTP	14	n.d.
Pyridoxal phosphate	9	n.d.
Adenosine	124	108
Adenine	116	n.d.
$MgCl_2$	104	125

* not determined.

Transhydrogenase is not induced until the end of the growth phase; activity is barely measurable in extracts from logarithmic phase cells. Induction is also related to the medium phosphate concentration (normally 0.33 mM), since raising the medium phosphate concentration to 3.3 mM results in a lower induction (10—30%) of transhydrogenase in stationary phase cells. Growth on glucose or gluconate-containing media causes a similar induction of the enzyme in the stationary phase as for growth on lactate-containing media.

If transhydrogenase has a role linking NADH formed by catabolism with NADPH generation for biosynthesis, it might be expected that it would be maximally induced during growth, when biosynthetic activity is greatest. Thus, induction during the stationary phase is unexpected. It is possible that, in the absence of growth substrates utilizing NAD-linked dehydrogenases, endogenous substrates associated with NADP-linked dehydrogenases could thereby be capable of providing NADH for respiration and ATP synthesis. However, the reason why the induction level of transhydrogenase is affected by the medium phosphate concentration is obscure.

Energy-linked transhydrogenase

An extensive series of experiments have failed to detect the presence of energy-linked transhydrogenase. These experiments, using published techniques [2—6] were uniformly unsuccessful when using the cell free extract, particulate or supernatant fractions at a variety of temperatures and pH values and in the

presence of various combinations of Mg^{2+}, dithiothreitol, EDTA, different buffers, etc. and attempting to drive the reaction by respiration or added energy source (ATP, phospho*enol*pyruvate, pyrophosphate, acetyl phosphate, GTP, CTP, etc.).

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Note added in proof (received October 26th, 1976)

The growth conditions for induction of transhydrogenase by *B. natriegens* are similar to those expected to cause induction of alkaline phosphatase, which could possibly act on NADP(H). This is very unlikely to occur here, because of the effect of glutathione plus glutathione reductase (Fig. 1) indicating NADPH as the end-product. In addition, we have incubated NADP(H) with extracts under the conditions given under Materials and Methods, followed by chromatography on cellulose plates, developing with 66 : 1 : 33 isobutyric acid/ammonia/water. No detectable conversion to NAD(H) occurred.

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