STRUCTURAL ASPECTS OF THE MEMBRANE-BOUND *ESCHERICHIA COLI* PYRIDINE NUCLEOTIDE TRANSHYDROGENASE (EC 1.6.1.1)

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1. Introduction

Escherichia coli, when grown on complex media containing high levels of amino acids, produce low levels of pyridine nucleotide transhydrogenase (EC 1.6.1.1) both energy-linked and independent [1,2]. Washing and replacement of such cells into a glucose minimal medium resulted in the 'de novo' synthesis of both transhydrogenase activities after 3 h [2]. Incorporation of chloramphenicol or omission of glucose in the induction medium resulted in no increase in activity. We have here used this induction system, in which a 5–10-fold increase in transhydrogenase is produced, to label newly synthesized polypeptides. By incorporating [^{3}H]casamino acids in the initial growth phase and non-repressive levels of [^{14}C]leucine in the induction phase, then partially purifying the transhydrogenase by slight modifications of a described procedure, we have been able to define the synthesized polypeptides on the basis of their high ^{14}C/^{3}H ratios after analysis on polyacrylamide gels in the presence of SDS.

2. Materials and methods

The preparation of a lipid-depleted *E. coli* NAD(P)$^+$ transhydrogenase has been reported [3]. This involved deoxycholate extraction of membranes from glucose-grown *E. coli* followed by DEAE-cellulose chromatography in the presence of Triton X-100. The enzyme was depleted of lipid by further treatment with cholate and ammonium sulphate. This preparation was activated by various phospholipids, in particular *E. coli* cardiolipin and phosphatidylglycerol. Similar preparations from glucose–yeast extract-grown cells could also be stimulated by phospholipids, but the maximal activity was only 10% of that in preparations from glucose-grown cells (fig.1). This differential in the level of activity was used as a means of analyzing the biosynthesis of the enzyme [2]. *E. coli* strain W6

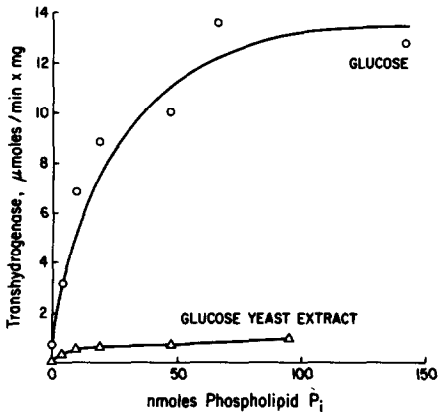

Fig.1. Stimulation of transhydrogenase isolated from normal and repressed *E. coli* membranes by phospholipid. The lipid-depleted enzyme was isolated from membranes of glucose- and glucose–yeast extract-grown cells as in [1]. The enzyme was then reactivated by preincubation for 5 min with varying levels of *E. coli* lipids before assaying in the acetyl pyridine NAD$^+$ reduction assay.

Abbreviations: AcPyNAD$^+$, acetyl pyridine-NAD$^+$; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; EDTA ethylenediamine tetra-acetic acid sodium salt; DOC, potassium deoxycholate

(a proline requiring auxotroph) was grown on complex media containing 500 μCi [^{3}H]casamino acids made to 1% with other unlabeled amino acids. The cells (250 ml) were grown to ~150 Klett units, then harvested, washed in minimal media, and then resuspended in 250 ml glucose–minimal medium [4], containing 50 μCi [^{14}C]leucine (spec. act. 270 mCi/mmol).

After 3 h of growth, the transhydrogenase activity was fully induced. The cells were harvested and the enzyme partially purified from the membranes by deoxycholate extraction and chromatography on DEAE–Sepharose in the presence of Triton X-100. This step was essentially the same as in [3], except for the introduction of a 150 mM NaCl wash prior to washing with the salt gradient, and the incorporation of glycerol into the column buffers. These steps were introduced to reduce a 70 kilodalton contaminant which appears to be related to a preceding flavoprotein on the column and to further stabilize the activity. The combined active eluates were then concentrated on an Amicon PM30 membrane, and dialyzed for 2 h against the buffer used for charging the column. The preparation was then passed over a second DEAE–Sepharose column (1 ml) to further concentrate the protein and to wash off the excess Triton, which concentrates on ultrafiltration. This was done by first washing out the high levels of Triton X-100 with starting buffer, followed by rapid elution with starting buffer containing 0.5% Triton X-100 and 500 mM NaCl. The concentrated fractions were then dialyzed against 10 mM phosphate buffer (pH 7) treated with SDS and mercaptoethanol, and subjected to SDS–gel electrophoresis. In most cases the polypeptides were alkylated with *N*-ethylmaleimide (100 mM) prior to electrophoresis in order to reduce occurrence of disulfide interchange, since studies with the membrane-bound enzyme indicate it has a highly reactive –SH component. Samples were electrophoresed on 7.5% acrylamide gels (Weber and Osborne).

The reduction of acetyl pyridine NAD$^+$ by NADPH was measured as in [4] except that 1 mM dithiothreitol was included in all assays. The reduction of NAD$^+$ by NADPH and NADP$^+$ by NADH were carried out using regenerating systems for NADPH (isocitrate dehydrogenase) and NADH (alcohol dehydrogenase), respectively [5] in the presence of 2 mM KCN.

NADH- and NADPH-ferricyanide reductase activities were assayed by the decrease in extinction at 420 nm of a 3 ml solution containing 300 μmol potassium phosphate (pH 7.0), 3 μmol K_3 Fe(CN)$_6$ and 0.3 μmol NADPH or NADH.

Radiolabelled L-[^{3}H]casamino acids and [^{14}C]-leucine (spec. act. 270 mCi/mmol) were obtained from the New England Nuclear Corp. Nucleotides were purchased from the Sigma Chemical Co.

3. Results and discussion

Growth of the *E. coli* under such conditions as to enable dual labeling of the polypeptides involved in transhydrogenation was outlined in section 2, along with a rationale for the procedures. The analysis of the isolated labeled transhydrogenase fraction on SDS–PAGE (fig.2a,b) indicated that the band with the highest ^{14}C/^{3}H ratios corresponded to a polypeptide of 94 kilodaltons. This high molecular weight component is comparable in size to the pyridine nucleotide

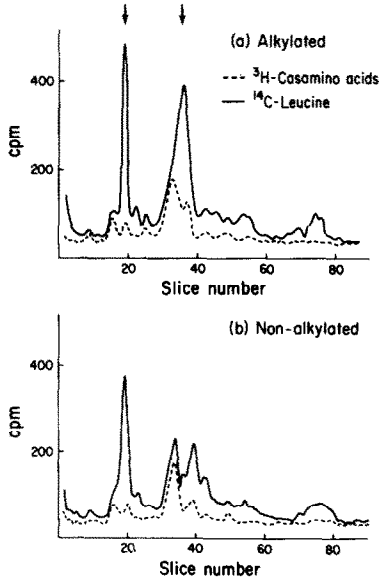

Fig.2. SDS–gel electrophoretic analysis of dual-labeled transhydrogenase preparation on 7.5% polyacrylamide gels (Weber and Osborne). Dual labeled transhydrogenase fraction (28 μg) after DEAE–Sepharose was digested with SDS and mercaptoethanol for 2 min at 100°C. (a) After alkylation with 100 mM *N*-ethylmaleimide. (b) Without alkylation. (——) [^{14}C]leucine; (- - -) [^{3}H]casamino acids.

transhydrogenase recently isolated from beef heart mitochondria [6,7]. A single polypeptide of 90–120 kilodaltons was independently isolated by two groups and shown to catalyze pyridine nucleotide transhydrogenation upon reconstitution into liposomes [6,8]. Similarities in structural aspects between the *E. coli* and mitochondrial enzymes may be expected on the basis of their similar kinetic properties and sensitivity to site-directed inhibitors and proteases [2,9]. The dual labeling studies, however, also indicate the presence of component(s) in the region of 50 kilodaltons which is synthesized together with the transhydrogenase as evidenced by a second band with a high $^{14}C/^{3}H$ ratio. Initial analyses indicate that in fact two components of almost identical molecular weight may exist in this region (data not shown). The detection of a band in this region was most perceptible upon alkylation of the polypeptides digested with SDS and mercaptoethanol as seen in fig.2a. The presence in the enzyme of a polypeptide containing a reactive sulfhydryl component would be consistent with earlier studies with the membrane-bound enzyme [3,10]. Two types of –SH groups were shown to be present in the enzyme which had differing sensitivities to sulphydryl modifying agents [10]. These sulphydryl groups seemed to be associated with the nucleotide binding sites. Reactive –SH groups have also been identified in the beef heart mitochondrial enzyme [11,12].

Fractionation of the lipid-depleted enzyme described [3] on agarose A50M in 50 mM Tris–SO_4 (pH 7.8) containing 10% glycerol, 1 mM dithiothreitol, 1 mM EDTA and 0.1% deoxycholate resulted in further purification of the enzyme. In general, two overlapping peaks of activity were obtained, which could not be easily resolved, with peaks at ~2 and 3-times the void volume. No activity was detectable in the void volume itself. Analysis of the alkylated polypeptides present in pooled active fractions, on SDS–PAGE (fig.3) again revealed the 94 kilodalton subunit and also the component(s) in the region of 50 kilodaltons, further adding weight to the relationship of the latter to the transhydrogenase activity.

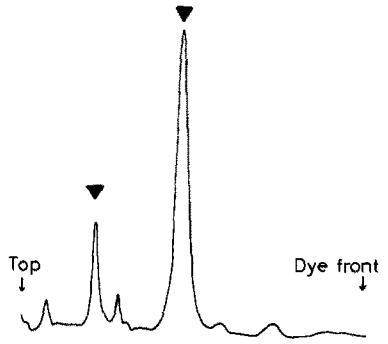

Fig.3. SDS–gel electrophoretic analysis of the agarose fraction of transhydrogenase on 7.5% polyacrylamide gels (Weber and Osborne). Agarose fraction (19 μg) was digested with SDS and mercaptoethanol for 2 h at 37°C and then applied to 7.5% acrylamide gel. The sample was alkylated with 100 mM *N*-ethylmaleimide and digested. The gel was stained for protein with Coomassie brilliant blue.

Table 1 indicates the type of purification achieved with the enzyme at this stage. The agarose enzyme has a basal activity in reduction of $AcPyNAD^+$ by NADPH of 2–5 μmol/min × mg which can be stimulated to 20–24 μmol/min × mg on direct addition of *E. coli* phospholipids. Lysolecithin is also relatively efficient in restoring activity. Incorporation

Table 1
Purification of *Escherichia coli* $NADP^+$ transhydrogenase

Fraction	Vol. (ml)	Protein (mg/ml)	Total protein (mg)	Total act. (μmol/min)	Spec. act. (μmol/min × mg)
Membranes	50	6.15	307	36.7	1.2
DOC extract	50	2.85	143	31.2	2.2
DEAE-Sepharose + cholate/$(NH_4)_2SO_4$	3	1.95	5.85	7.1	12.2[a]
Agarose A50M	10	0.27	2.7	5.5	20.2[a]

[a] In the presence of *E. coli* phospholipids

Activities were measured in terms of the reduction of $AcPyNAD^+$ by NADPH

Table 2
Activities present in the purified transhydrogenase preparation

	−Phospho-lipids	+ 20 μg Lyso-lecithin	+ *E. coli* phospholipids
	(μmol oxidised/min × mg)		
Transhydrogenases			
NADPH-AcPyNAD$^+$	2.1	12.9	23.3
NADPH-NAD$^+$	0.67	5.83	3.85
NADH-NADP$^+$	0.17	0.72	0.38
Ferricyanide reductases			
NADH-Fe(CN)$_6^{3-}$	<0.01	<0.01	<0.01
NADPH-Fe(CN)$_6^{3-}$	0	0	0

Activities were measured as in section 2. The agarose fraction was used for these experiments

into liposomes and activation of transhydrogenation by uncouplers as described for the beef heart enzyme is being investigated at present [6,8]. The agarose fraction was also capable of catalyzing the non-energy-linked reduction of NAD$^+$ by NADPH, and at high protein levels, reduction of NAD(P)$^+$ by NADH (table 2). The NAD$^+$ reduction by NADPH, as expected, was more rapid than the non-energy-linked forward direction by a factor of 5–10. NADH and NADPH-ferricyanide reductase activities were not detected in the fraction, either in the absence or presence of added phospholipids. At this stage, there appeared to be no enrichment in flavin or non-heme iron, leaving open the question as to the identity of its functional group(s) in the redox reaction.

The relationship between the 94 kilodalton and 50 kilodalton components is at present unclear and is the subject of further study. The possibility of the higher molecular weight species being an exceptionally stable dimer of the lower molecular weight species is not being ruled out. Nor is it unreasonable that the 50 kilodalton component(s) is a cleavage product of the 94 kilodalton subunit. This would be compatible with the high trypsin sensitivity of the *E. coli* enzyme, and with the absence of this species in the mitochondrial enzyme, which is more resistant to proteolytic digestion [9]. Yet another possibility is that the enzyme resembles that present in *Rhodospirillum rubrum* which consists of a membrane-bound and soluble component. The latter is known to be highly sensitive to sulphydryl reagents [13].

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