Expression of the cloned subunits of *Escherichia coli* transhydrogenase from separate replicons

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The *pntA* and *pntB* genes of *Escherichia coli*, encoding the α- and β-subunits of the pyridine nucleotide transhydrogenase, were cloned individually in two different compatible plasmids into *Escherichia coli* mutants lacking transhydrogenase activity. Energy-linked and non-energy-linked transhydrogenase activities were produced only in cells carrying both plasmids thus showing that the products of both genes are required for the formation of an active enzyme. ATP-energized transhydrogenase activity was not increased in cells containing amplified levels of the transhydrogenase when the cell membrane ATPase was also amplified. It is suggested that the excess transhydrogenase is effectively uncoupled from the ATPase by compartmentalization in the cell.

Transhydrogenase *ATPase* *Cloned subunit* pnt *gene* *Reconstitution* *ATP energization*

1. INTRODUCTION

Pyridine nucleotide transhydrogenase found in the cytoplasmic membrane of *Escherichia coli* and in the inner mitochondrial membrane catalyzes the transfer of a hydride ion equivalent between reduced and oxidized forms of NAD and NADP. The rate of reduction of NADPH by NADH is increased several fold on membrane energization by respiration or ATP hydrolysis [1].

Recently we have cloned the *pnt* (pyridine nucleotide transhydrogenase) gene of *E. coli* into a bacterial plasmid [2]. Growth of cells containing the plasmid resulted in up to 70-fold amplification of transhydrogenase activity and in the appearance of two polypeptides, of M_r 50000 and 47000, in the cell membrane. The enzyme purified from the cytoplasmic membrane of *E. coli* also contained these two polypeptides [3]. However, the bovine mitochondrial transhydrogenase consists of a single polypeptide chain of M_r 97000–120000 [4,5]. Moreover, Voordouw et al. [6] found an immunologically cross-reactive polypeptide of the same relative molecular mass in partially purified preparations of the *E. coli* transhydrogenase when using antibodies directed against the mitochondrial transhydrogenase. Liang and Houghton [7] have found polypeptides of M_r 94000 and 50000 in a partially purified preparation of the *E. coli* enzyme.

These results raise the possibility that the two polypeptides found in our transhydrogenase preparations are proteolytic fragments of a larger enzyme. Reconstitution of the two polypeptides to form an active transhydrogenase would rule out this possibility. This has been achieved in the present paper. We also describe the effect on ATP-energized transhydrogenation of amplification of membrane ATPase activity in the cell.

2. MATERIALS AND METHODS

E. coli (strain JM83) was used for propagation of recombinant plasmids and was described in [2]. *E. coli* (strain AB1450) contains the transposon Tn5 in the *pnt* locus and completely lacks transhydrogenase activity [2]. The vectors used were: pUC13 (amp^r), pDC9 (amp^r), pDC11 (amp^r), pDC23 (amp^r), which have been described previously [2], pACYC184 (amp^r, cm^r) [8], and

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pRPG54 (cm^r) [9]. The procedures described by Maniatis et al. [10] were used for the isolation of plasmid DNA, ligations and transformations. *E. coli* carrying plasmids was routinely grown in LB medium [11] or plated (LB medium with 1.5% agar) at 37°C with the appropriate antibiotic (one or more of the following: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 30 μg/ml).

Plasmid pDC50 was constructed by digesting plasmid pDC23 with restriction endonucleases *Hin*dIII and *Bam*HI. The released insert, containing the gene encoding the 47 kDa polypeptide, was inserted into the *Hin*dIII and *Bam*HI sites of plasmid pACYC184, transformed into JM83, and grown on LB-agar plates with chloramphenicol. Colonies which grew on these plates were selected and microscreened. The plasmid pDC50 contained the 3.6 kb insert.

Membranes were prepared by suspending cells in 50 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol, 1 mM EDTA and lysing by passage through an ice-cold French pressure cell at 1400 kg/cm^2. Unbroken cells were removed by centrifugation at 12000 × *g* for 10 min at 4°C. The supernatant was centrifuged at 180000 × *g* for 1 h at 4°C. The membranes were resuspended in buffer and recentrifuged. Energy-independent transhydrogenase activity was assayed using 3-acetylpyridine adenine dinucleotide and NADPH [3]. Energy-dependent transhydrogenase activity was measured by the reduction of NADP by NADH using a regenerating system for NADH [12]. ATPase was assayed by measuring the release of P_i [13].

3. RESULTS AND DISCUSSION

Fig.1A shows the partial restriction map of pDC11 which carries a 4.05 kb DNA insert with both *pntA* and *pntB* genes. The partial restriction maps of the plasmids pDC9 and pDC23, which carry intact *pntA* and *pntB* genes, respectively, are shown in fig.1B and C. The plasmid containing the *pntA* gene has a 3.2 kb insert in which part of the *pntB* gene has been removed from the 4.05 kb insert of pDC11. The plasmid containing the *pntB* gene has a 0.45 kb deletion in the *pntA* gene of the same 4.05 kb insert. A full description of these plasmids is given in [2]. Plasmid pDC50 carries the same insert as pDC23 but in pACYC184 instead of pUC13.

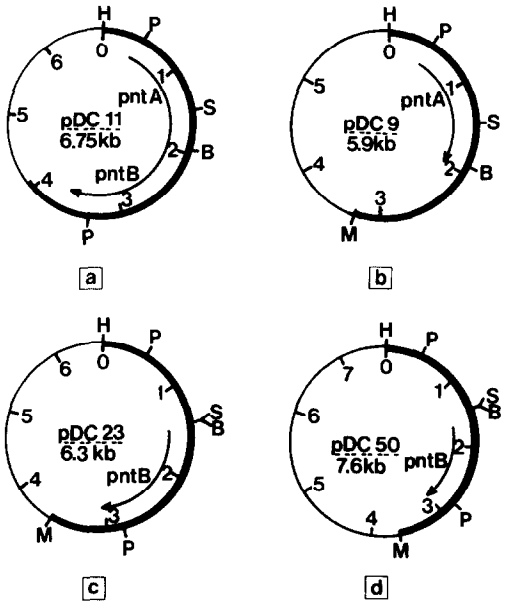

Fig.1. Partial restriction maps of plasmids. (a) pDC11 with intact *pntA* and *pntB* genes; (b) pDC9 with intact *pntA* gene; (c) pDC23 with intact *pntB* gene; (d) pDC50 with intact *pntB* gene. B, *Bst*EII; H, *Hin*dIII; M, *Bam*HI; P, *Hpa*I; S, *Sal*I. Heavy lines indicate the insert DNA. The approximate locations of intact *pntA* and *pntB* genes are indicated.

E. coli carrying any one of the plasmids pDC9, pDC23 or pDC50 did not contain an active transhydrogenase (table 1). Membranes from *E. coli* carrying pDC9 contained the 50 kDa polypeptide whereas those from cells with pDC23 or pDC50 contained the 47 kDa polypeptide [2]. However, membranes from *E. coli* carrying both plasmids pDC9 and pDC50 exhibited high levels of transhydrogenase activity (table 1) and contained both polypeptides (fig.2). The relative amount of the 50 and 47 kDa polypeptide reflects the difference in plasmid copy numbers. The lower transhydrogenase activity of these cells when compared with those carrying pDC11 is also due to the lower copy number of pDC50. These results show that the *E. coli* transhydrogenase consists of two subunits and confirm our previous suggestion [3] that the high-M_r polypeptide observed by Liang and Houghton [7] and Voordouw et al. [6] is an aggregate of transhydrogenase subunits.

Table 1

Complementation of chromosomal *pnt*::Tn*5* by various *pnt* alleles on plasmids

Strain	Transhydrogenase activity in membranes (μmol/min per mg protein)
AB1450	0.044
AB1450 *pnt*::Tn*5*	0
AB1450 *pnt*::Tn*5* pUC13	0
AB1450 *pnt*::Tn*5* pDC9	0
AB1450 *pnt*::Tn*5* pDC23	0
AB1450 *pnt*::Tn*5* pDC11	1.98
AB1450 *pnt*::Tn*5* pDC9, pDC50	1.04

E. coli strain AB1450 *pnt*::Tn*5* was transformed with plasmids pUC13, pDC9, pDC11, pDC23 and/or pDC50 and then grown in LB media supplemented with kanamycin (25 μg/ml) and ampicillin (50 μg/ml). Membranes were prepared and assayed for transhydrogenase activity as described in section 2

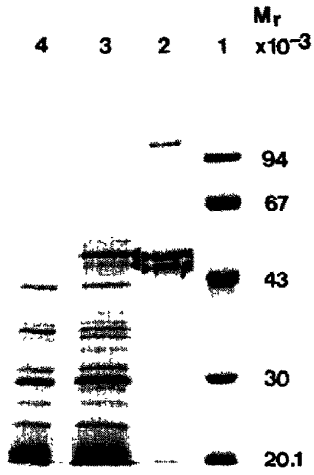

Fig.2. SDS-polyacrylamide gel electrophoresis of membranes prepared from *E. coli* AB1450 (*pnt*::Tn*5*) and AB1450 (*pnt*::Tn*5*) pDC9, pDC50. Cells were grown in LB medium supplemented with kanamycin (30 μg/ml). In addition, the medium for the plasmid-containing strain contained ampicillin (50 μg/ml) and chloramphenicol (30 μg/ml). Electrophoresis on 10% (w/v) polyacrylamide gels was carried out as in [2]. Lanes: 1, M_r markers; 2, purified transhydrogenase; 3, membranes of AB1450 (*pnt*::Tn*5*) pDC9, pDC50; 4, membranes of AB1450 (*pnt*::Tn*5*).

ATP-energized transhydrogenase activity was also restored to cells carrying both plasmids. Addition of ATP caused a 3-fold stimulation in the rate of reduction of NADP by NADH in membrane preparations from 0.14 to 0.44 units/mg protein. A 7-fold stimulation (0.009 to 0.064 units/mg protein) of transhydrogenation following addition of ATP is observed in strain JM83. The possibility that the rate of ATP-energized transhydrogenation in membranes containing highly amplified levels of transhydrogenase might be limited by the rate of ATP-dependent energization was examined. *E. coli* AB1450 (*pnt*::Tn*5*) containing pDC11 was transformed with pRPG54, which contains the *unc* operon for the synthesis of the cell membrane ATPase [9]. Transformation resulted in an increase in membrane ATPase activity from 0.24 to 1.24 units/mg protein. However, as before, ATP stimulated transhydrogenation only 3-fold from 0.11 to 0.35 units/mg protein. This suggests that the excess transhydrogenase in membranes from amplified cells must be uncoupled from ATP-dependent energization. We have observed tubular-like structures in cells overproducing transhydrogenase similar to those which are formed during overproduction of fumarate reductase [14]. It is possible that the transhydrogenase in membranes derived from the tubular structures during French pressing might lack ATPase activity. This would result in an uncoupling of ATPase and transhydrogenase activities. This possibility is being examined.

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