Enzymological and physiological consequences of restructuring the lipoyl domain content of the pyruvate dehydrogenase complex of *Escherichia coli*

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The core-forming lipoyl acetyltransferase (E2p) subunits of the pyruvate dehydrogenase (PDH) complex of *Escherichia coli* contain three tandemly repeated lipoyl domains although one lipoyl domain is apparently sufficient for full catalytic activity *in vitro*. Plasmids containing IPTG-inducible *aceEF-lpdA* operons which express multilip-PDH complexes bearing one N-terminal lipoyl domain and up to seven unlipoylated (mutant) domains per E2p chain, were constructed. Each plasmid restored the nutritional lesion of a strain lacking the PDH complex and expressed a sedimentable PDH complex, although the catalytic activities declined significantly as the number of unlipoylated domains increased above four per E2p chain. It was concluded that the extra domains protrude from the 24-meric E2p core without affecting assembly of the E1p and E3 subunits, and that the lipoyl cofactor bound to the outermost domain can participate successfully at each of the three types of active site in the assembled complex. Physiological studies with two series of isogenic strains expressing multilip-PDH complexes from modified chromosomal *pdh* operons (*pdhR-aceEF-lpdA*) showed that three lipoyl domains per E2p chain is optimal and that only the outermost domain need be lipoylated for optimal activity. It is concluded that the reason for retaining three lipoyl domains is to extend the reach of the outermost lipoyl cofactor rather than to provide extra cofactors for catalysis.

Keywords: pyruvate dehydrogenase complex, lipoyl acetyltransferase, lipoyl domains, protein engineering, metabolic engineering

INTRODUCTION

Escherichia coli uses three enzymes for catalysing pyruvate oxidation under different physiological conditions. The pyruvate dehydrogenase (PDH) complex operates during aerobic respiratory growth but its function is increasingly adopted by pyruvate formate-lyase (PFL) under anaerobic conditions. Thus, the PDH complex and PFL are both functional during anaerobic respiratory growth with nitrate (Kaiser & Sawers, 1994), whereas PFL takes over completely during fermentative growth. In contrast, pyruvate oxidase (PoxB), which

converts pyruvate directly to acetate rather than to acetyl-CoA, is an aerobic stationary-phase enzyme (Chang *et al.*, 1994). These enzymes control the flux of glycolytic carbon into growth-related products, including the products of heterologous gene expression, as well as normal metabolites. The ability to manipulate the synthesis or activity of these enzymes offers the possibility of redirecting substrate carbon into biotechnologically useful products. It could also provide a means of redirecting substrate carbon away from acetate, which accumulates at high cell density and by uncoupling membrane potential, is a major cause of poor productivity and cell death (Cutayer & Poillon, 1989; Konstantinov *et al.*, 1990). One strategy for manipulating pyruvate metabolism is to replace the pyruvate-inducible regulatory region of the *pdh* operon (*pdhR-aceE-aceF-lpdA*; Fig. 1) by the IPTG-regulated

Abbreviations: D, dilution rate; E1p, pyruvate dehydrogenase, E2p, lipoyl acetyltransferase; E3, lipoamide dehydrogenase; PDH, pyruvate dehydrogenase (complex).

Fig. 1. Construction of a dual replicon (pGS641) containing two fragments of the *aceF* region encoding tandem wild-type and mutant lipoyl domains. Restriction map of the *pdhR-aceEF-lpdA* region (not to scale) showing the location of the *KpnI-SphI* cassettes encoding lipoyl domains with wild-type (+) and mutant (-) lipoyl-binding sites (different origins are denoted by the shaded boxes: *lip1*, open; *lip2*, hatched; and *lip3*, filled) and the associated linkers (zig-zag lines). The *KpnI-EcoRI* insert (+ - -) of pGS640 was derived by sub-cloning the 300 bp *AccI* fragment of pGS585 into pGS584 in the desired orientation, and then sub-cloning the *KpnI-HindIII* fragment of pGS587 into pUBS1 to pick up a flanking *EcoRI* site for ligation into the dual replicon, pPOD6. The *XhoI-SphI* insert (- - -) of pGS641 was constructed in pGS586 by replacing the 700 bp *AccI-SphI* fragment of pGS585 by the corresponding fragment of pGS584 and then ligated between the *BglII* and *SphI* sites of pGS640, where the *BglII* site was unexpectedly lost. Abbreviations/symbols: □, *AccI*; ▼, *BclI*; B, *BamHI*; Bg, *BglII*; E, *EcoRI*; H, *HindIII*; K, *KpnI*; P, *PstI*; Sa, *Sall*; Sm, *SmaI*; Sp, *SphI*; St, *SstI*; X₁, *XhoI*; X₂, *XhoII*.

tac promoter so as to control synthesis of the PDH complex by the concentration of added IPTG (Cassey *et al.*, 1996; Guest *et al.*, 1996). An alternative strategy is to replace the genes encoding catalytic subunits by genetically manipulated variants, thus to alter the

efficiency of the PDH complex (Davé *et al.*, 1995). The second approach has now been extended to include further modifications of the PDH complex and at the same time, to use physiological criteria for assessing structure-function relationships of the complex.

The PDH complex of *E. coli* catalyses the NAD⁺-linked oxidative decarboxylation of pyruvate to acetyl-CoA by three integrated reactions (Guest *et al.*, 1989; Perham, 1991; Mattevi *et al.*, 1992). It contains three enzymic subunits encoded by the *aceE*, *aceF* and *lpdA* genes of the *pdh* operon: pyruvate dehydrogenase (E1p, EC 1.2.4.1), lipoate acetyltransferase (E2p, EC 2.3.1.12) and lipoamide dehydrogenase (E3, EC 1.8.1.4), respectively (Fig. 1). Multiple copies of the dimeric E1p and E3 components are assembled on the respective edges and faces of a cubic core containing 24 E2p subunits, the overall chain stoichiometry (E1p:E2p:E3) being approximately 1:0:1:0:0:5. The E2p subunits contain three similar lipoyl domains (lip) connected to each other and to a small E3-binding domain (E3bd), by conformationally mobile linkers (xxxx, where x denotes about five residues), and a C-terminal catalytic domain (CAT):

NH₂-lip1-xxxx-lip2-xxxx-lip3-xxxx-
E3bd-xxx-CAT-COOH

The CATs aggregate to form a 24-meric inner core from which the lipoyl domains protrude. Each lipoyl domain contains a covalently bound lipoyl cofactor that is attached post-translationally to a prominent lysine residue either by lipoate protein ligase which uses pre-formed lipoate and ATP, or by an alternative mechanism which takes lipoyl groups directly from the *de novo* biosynthetic pathway (Green *et al.*, 1995; Morris *et al.*, 1995). The lipoyl cofactors project from the inner core and approach the acetyltransferase active sites from outside the core whereas CoA enters the catalytic channel from within the core (Mattevi *et al.*, 1992). All of the lipoyl cofactors are functional and the mobility inherent in the 'domain-linker' organization and the lipoyllysine 'swinging arm' is presumed to facilitate their reductive acetylation, deacetylation and re-oxidation at the E1p, E2p and E3 catalytic sites in the assembled complex.

The numbers of lipoyl domains per E2p chain varies from one to three in different 2-oxo acid dehydrogenase complexes and it is not clear why three are retained by the PDH complexes of *E. coli* and other Gram-negative bacteria, e.g. *Azotobacter vinelandii* (Schulze *et al.*, 1992). Site-directed deletion of the *lip* segments encoding one or two lipoyl domains has no detectable effect on specific activity or active-site coupling, but the catalytic activity is abolished when all three *lip* segments are deleted or when the lipoyl domains are rendered unlipoylatable by lipoyl-Lys → Gln substitutions (Guest *et al.*, 1985; Allen *et al.*, 1989). No mandatory order of reductive acetylation is apparent because complexes containing mixtures of wild-type and mutant lipoyl domains (+ - ; - + ; + + -) are fully active, although some impairment is associated with the - - + arrangement (Allen *et al.*, 1989). Activity is also impaired (but not abolished) by increasing the lipoyl domain content to between four to nine per E2p chain, possibly due to under-lipoylation of the domains participating in catalysis and to steric interference from unlipoylated domains (Machado *et al.*, 1992). An important feature

contributing to a high catalytic efficiency is the conformational mobility conferred by the linker sequences. This is apparent from the impairment that accompanies the shortening or replacing of the residual lipoyl linker of a 1lip-PDH complex, and the enhancement afforded by incorporating a polyproline linker (Miles *et al.*, 1988; Turner *et al.*, 1993). High-field H¹-NMR studies with variants containing from zero to nine lipoyl domains per E2p chain gave the first indication that the mobility of the lipoyl linkers relative to the domains, and hence the catalytic efficiency, is highest in the wild-type complex (Machado *et al.*, 1993). The superiority of the 3lip-PDH complex has since been confirmed in physiological studies with stable isogenic strains that express 1lip-, 2lip- and 3lip-PDH complexes, in which it was concluded that lowering the lipoyl domain content adversely affects growth rate and yield (Davé *et al.*, 1995). In the present report, the physiological consequences of increasing the number of lipoyl domains from three to nine per E2p chain, and the effects of inserting up to seven unlipoylatable (mutant) domains between a wild-type N-terminal lipoyl domain and the E3-binding domain, were investigated. The results show the extent to which a single lipoyl cofactor can be distanced from the cognate active sites yet retain activity, and they help to explain why the wild-type E2p chain retains three lipoyl domains.

METHODS

Bacteria, plasmids and cloning procedures. The strains of *E. coli* K-12 were: W3110 (*iclR trpR*), the prototrophic strain; JRG2390 (*aceF::kan^R*), a derivative of W3110 in which the *aceF* gene was inactivated by a net deletion of two *lip* segments and insertion of a *kan^R* cassette into the residual *lip* segment (Davé *et al.*, 1995); JRG2931-2933 (1lip-3lip), derivatives of a spontaneous Nal^R mutant of JRG2930 in which the *aceF::kan^R* region was replaced by *aceF* genes encoding E2p subunits with one to three lipoyl domains (Davé *et al.*, 1995); JRG3134-3139 (4lip-9lip) and JRG3162-3168 (2lip-8lip), analogous derivatives of JRG2930 containing *aceF* genes with multiple *lip* segments (this work); DH5α (*ΔlacU169 φ80lacZΔM15 hsdR17 endA1 recA1*), the routine transformation host (Sambrook *et al.*, 1989); JC7623 (*recB21 recC22 sbcB15 sbcC201*), for chromosomal gene replacement (Oden *et al.*, 1990); GM242 (*dam-3 recA1*), for producing plasmids sensitive to *BclI* digestion; and JRG1342 [*Δ(aroP-pdhR-aceEF-lpdA)18 pox recA1*], for over-producing modified PDH complexes and for nutritional tests (Guest *et al.*, 1985).

The dual replicon pPOD6 containing replication origins from pACYC184 (A) and pIJ2925 (B) linked to Km^R and Ap^R determinants (respectively) and two different multi-cloning sites (Fig. 1), was kindly provided by A. F. C. Perry and N. J. Brown (University of Birmingham, unpublished).

The *KpnI*-*SphI* cassettes containing multiple *lip* segments (*aceF* sub-genes) were constructed from those of pGS231 (+ -) and pGS234 (- - +) (Allen *et al.*, 1989) after transfer to the intermediary vector (pT7T3 18U, Pharmacia) to generate pGS584 (+ -) and pGS585 (- - +), respectively (Fig. 1). These plasmids were used as sources of the *KpnI*-*EcoRI* (+ - -) and *XhoII*-*SphI* (- - -) cassettes of pGS562 and pGS586 (respectively) which were transferred sequentially to pPOD6 to generate pGS641 via pGS640, as summarized in Fig. 1. Plasmid pGS641 was maintained in DH5α with selection

Fig. 2. Construction of expression plasmids encoding PDH complexes with two to eight lipoyl domains per E2p chain. Kanamycin-resistant resolvants of pGS641 were generated by partial digestion with *Bcl*I (▼), or complete digestion with *Hind*III (3lip only) and re-ligation to give pGS642–645. A derivative of pGS645 lacking the vector *Sal*I/*Acc*I site (pGS645a), was constructed by *Sal*I cleavage, end-filling and re-ligation, linearized with *Kpn*I, and partially digested with *Acc*I (□) to yield fragments that can be combined to form plasmids encoding higher orders of lipoyl domains (pGS646–648), as indicated. The *Kpn*I–*Sph*I cassettes from each intermediary plasmid (pGS642–648) were transferred to pGS501, a *P*_{tac} expression plasmid and cassette-replacement receptor for the *aceEF-lpdA* operon, to generate the corresponding set of expression plasmids pGS662–668. Abbreviations/symbols as in Fig. 1.

for both antibiotic markers to prevent segregation of the two replicons by internal crossing-over between homologous regions of the sub-cloned segments. The expression plasmid, pGS501 (Russell *et al.*, 1992), in which an *aceE-aceF-lpdA* operon is expressed from the IPTG-inducible *tac* promoter (Fig. 2), served as receptor for the original and restructured *KpnI-SphI* cassettes of pGS642–648 to generate pGS662–668 (Fig. 2). Analogous expression plasmids containing operons with zero to nine wild-type *lip* segments were constructed previously (Machado *et al.*, 1992): pGS489 (0*lip*), pGS367 (1*lip*), pGS524 (2*lip*), pGS523 (3*lip*), pGS504 (4*lip*), pGS505 (5*lip*) and pGS556–559 (6*lip*–9*lip*). Other plasmids were: pUBS1, a derivative of pUC19 containing the multi-cloning site from pBluescriptSK (Dr G. Murphy, Institute of Plant Science Research, Cambridge) used in sub-cloning; pUC4K+, a derivative of pUC4K (Pharmacia) in which the *kan^R* gene is flanked by two multi-cloning sites from pUC19, used as the source of the *kan^R* cassette; and pMAK705 (Ap^R, Cm^R), thermo-sensitive replicon used for chromosomal gene replacement (Hamilton *et al.*, 1989). Standard molecular genetic procedures (Sambrook *et al.*, 1989) were used, JRG2930 and JRG1342 were transformed by electroporation, and transduction with P1vir1 was used for strain construction (Miller, 1972).

Media, nutritional tests and enzymology. Cultures were grown in L broth plus glucose (6 mM) supplemented appropriately with ampicillin (50 µg ml⁻¹) or kanamycin (20 µg ml⁻¹). Nutritional tests were performed with plates of glucose (11 mM) minimal media supplemented with acetate (2 mM), thiamin (10 µg ml⁻¹), L-methionine (10 µg ml⁻¹), ampicillin (25 µg ml⁻¹) and IPTG (20 µM), as required (Guest *et al.*, 1985). The over-production and purification of PDH complexes from transformants of JRG1342 involved induction with IPTG (60 µM), ultrasonic disruption, sedimentation from crude extracts and FPLC ion-exchange chromatography (Mono-Q), as described by Russell *et al.* (1992). Specific activities [in units (mmol NADH formed)⁻¹ mg protein⁻¹] of the PDH complexes, were calculated from the rates of pyruvate-dependent reduction of NAD⁺ (Russell & Guest, 1990); mean values for three independent preparations are quoted. *M_r* values and subunit stoichiometries were estimated by SDS-PAGE (0.1% SDS, 7.5% resolving gel, 4.5% stacking gel, with 3 µg protein in each lane) and densitometric analysis (Bio-Profil) after staining with Coomassie Brilliant Blue R250. The procedure for lipoylating purified PDH complexes with lipoyl protein ligase has been described by Brookfield *et al.* (1991) and Green *et al.* (1995).

Chromosomal replacement procedures. The chromosomal *aceF* gene of *E. coli* W3110 was replaced by altered genes containing multiple wild-type *lip* segments using derivatives of thermo-sensitive replicon, pMAK705 (Hamilton *et al.*, 1989). The procedure was similar to that used previously for lowering the number of *lip* segments (Davé *et al.*, 1995) except that a new series of pMAK705-derivatives having a larger *aceF-lpd* flanking region was used in order to improve the frequency of chromosomal replacement. The relevant plasmids were constructed by first cloning the 4.6 kb *KpnI-SalI* fragment of pGS367 (Russell *et al.*, 1992) containing a wild-type *aceEF-lpdA* segment into pUBS1 to provide the flanking *HindIII* site in pGS779, and then transferring the 2.32 kb *SphI-HindIII* *aceF-lpdA* fragment to pMAK705 to generate pGS780 (Fig. 3). The 1.3 kb *kan^R* cassette of pUC4K+ was inserted at the vector *BamHI* site to provide a convenient phenotypic marker in the product, pGS781, which was readily replaced by *KpnI-SphI* (*aceEF*) segments from the 'multilip' expression plasmids (pGS504, -505, -556–559) at the same time

Fig. 3. Construction of isogenic strains containing single copies of genetically modified *pdh* operons. The genes and promoters of the wild-type *pdh* operon in which the *aceF* gene includes three *lip* segments (L), are shown at the top. Derivatives of the pMAK705 (*rep-ts cml*) containing segments of the operon used in modifying the chromosomal *aceF* gene (numbered on the left) and the corresponding strains (numbered on the right), were constructed and used as described in Methods. The *kan^R* cassette is shaded and relevant restriction sites are: B, *BamHI*; H, *HindIII*; K, *KpnI*; Sa, *SalI*; and Sp, *SphI*.

restoring continuity to the *aceEF-lpdA* region (Fig. 3). This provided a series of appropriately modified thermo-sensitive replicons (pGS804–809) with which to generate the corresponding chromosomally replaced strains (JRG3134–3139, 4*lip*–9*lip*) by sequential co-integration into, and resolution from, the chromosome of JRG2930 (*aceF::kan^R Nal^S*), as described by Davé *et al.* (1995).

An analogous series of isogenic strains containing *aceF* genes with one wild-type *lip* segment and up to seven mutant *lip* segments, was made by the method of Oden *et al.* (1990). A transformation host, JRG3141 (*recBC sbcBC aceF::kan^R*), was first constructed by P1 transduction of JC7623 with JRG2930 (*aceF::kan^R*) as donor, and selecting a representative Km^R Ace⁻ transductant (the Ace⁻ phenotype is characterized by the inability to grow on glucose or succinate minimal media unless provided with an acetate supplement). Derivatives of JRG3141 containing the desired chromosomal replacements were recovered as Ace⁺ Km^S Ap^S products after transformation with pGS662–668 and designated JRG3141–3148. In the final step, the altered *aceF* genes were transferred from the JC7623 background to the W3110 background by selecting Ace⁺ (Km^S) transductants of JRG2930 (*aceF::kan^R Nal^S*) on glucose minimal medium, to provide the corresponding set of strains, JRG3162–3168.

Both sets of isogenic strains were numbered so that the multiplicity of *lip* segments in the chromosomal *aceF* gene is defined by the final digit of the strain designation. The chromosomal replacements were confirmed by PCR amplification of a diagnostic fragment containing the modified *lip* region using a bacterial colony as the source of template DNA and oligonucleotide primers corresponding to sequences at the 5' end of the *aceE* gene (S361, 5'-GTTGATGCTTC-TTATGTCGTGGTTGCGGC3'; coordinates 3635–3663,

Stephens *et al.*, 1983a) and within the *aceF* gene (S360, 3'CCACAATTGGAACGCTTTCACTTCCCCTGAC5'; coordinates 4804–4835, Stephens *et al.*, 1983b). In each case the amplified fragment was of the size predicted for the corresponding number of lipoyl domains.

Physiological methods. The media, growth conditions and analytical methods used in quantitative physiological studies with batch and glucose-limited continuous cultures, have been described by Davé *et al.* (1995) and Brooke *et al.* (1989). The specific growth rates ($\mu_{\max}$) were measured using cultures starting from single colonies from glucose minimal agar and developing successive overnight shake cultures of 10, 50 and 100 ml in glucose minimal medium (20 mM glucose; pH 6.95) to provide 100 ml inocula for 600 ml batch cultures in chemostat vessels operating at $\geq 50\%$ air saturation and 37 °C. Growth was estimated from the optical density at 430 nm (Pye Unicam SP6-250 spectrophotometer) and values for $\mu_{\max}$ were calculated from the doubling times. The quoted values are means from at least three independent cultures; deviation from the mean was no more than $\pm 5\%$. Pairwise competition experiments were performed in glucose-limited chemostat cultures (20 mM glucose; D 0.075 h⁻¹) with approximately equal numbers of Nal^R and Nal^S bacteria in the starting population, as described by Davé *et al.* (1995).

RESULTS

Construction of *pdh* operons encoding PDH complexes containing one wild-type and up to seven unlipoylatable lipoyl domains per E2p chain

The strategy devised for constructing plasmids that encode one wild-type lipoyl domain (+) and up seven mutant domains (–) is summarized in Figs 1 and 2. It involved manipulating pre-existing DNA fragments with (+ –) and (– – +) configurations to produce two derivatives, (+ – –) and (– – –), which were then

sequentially sub-cloned into the dual-replicon (pPOD6) to generate pGS641 (Fig. 1). Kanamycin-resistant resolvants of pGS641 containing two to five *lip* segments were then created by partial *Bcl*I digestion (or *Hind*III digestion in one case) followed by re-ligation, as shown in Fig. 2. Analogous plasmids containing six to eight *lip* segments were constructed by linearizing a derivative of the *5lip* plasmid (pGS645a) with *Kpn*I followed by partial digestion with *Bcl*I and the re-ligation of different pairs of sub-fragments (Fig. 2). The *Kpn*I–*Sph*I fragments from each of these intermediary plasmids (pGS642–648) were transferred to pGS501 to generate a set of P_{tac} expression plasmids encoding the corresponding PDH complexes (pGS662–668). The two series of plasmids were designated +/– to distinguish them from the +/+ series that contain multiple copies of wild-type lipoyl domains (Machado *et al.*, 1992). The numbers of *lip* segments and other predicted features of all types of intermediate and ultimate plasmids were thoroughly confirmed by restriction analysis. All of the +/– expression plasmids complemented the *Ace*[–] nutritional phenotype of an *aroP*–*pdhR*–*aceEF*–*lpdA* deletion strain (JRG1342) but only in the presence of IPTG (20 μ M). It would thus appear that the lipoyl cofactors bound solely to the outermost domains of the E2p chains in each of +/– PDH complexes confer sufficient enzymic activity to support the growth of the deletion strain.

Properties of +/– PDH complexes containing up to eight lipoyl domains

After induction with IPTG, all of the expression plasmids produced sedimentable PDH complexes which could be purified by the method developed for the

Table 1. Properties of PDH complexes with multiple lipoyl domains per E2p chain

The properties of the +/– series of purified PDH complexes are compared with those of the 1lip–PDH complex expressed by the receptor plasmid (pGS501) and the inactive 0lip–PDH complex (pGS489). M_r were calculated from the nucleotide sequences (without lipoic acid, Stephens *et al.*, 1983b) or estimated from the mobilities in SDS-PAGE (mean of three gels for three preparations). The predicted M_r for each lipoyl domain plus associated linker is approximately 11250 (innermost domain) and 10000–10400 (other domains). The specific activities [units (mg protein)⁻¹] are means for three preparations, the individual activities deviated < 15% from the quoted value.

Plasmid	Lipoyl domains		M_r of E2p chain			Increment in M_r per lipoyl domain	Specific activity of +/– PDH complex
	Total	E2p	Predicted	Apparent	Difference		
pGS489	0		34345	34000	–345	–	< 0.001
pGS501	1	+	45610	50600	4990	16600	14.5
pGS662	2	+ / 1 –	55592	65500	9908	14900	13.6
pGS663	3	+ / 2 –	65959	77200	11241	11700	11.8
pGS664	4	+ / 3 –	76327	90100	13773	20900	11.9
pGS665	5	+ / 4 –	86309	106000	19691	15900	10.2
pGS666	6	+ / 5 –	96677	116100	19423	10100	8.0
pGS667	7	+ / 6 –	106659	136800	30141	20800	4.9
pGS668	8	+ / 7 –	117027	151500	34473	14700	2.1

Fig. 4. SDS-PAGE analysis of purified $\pm$ PDH complexes containing one to eight lipoyl domains per E2p chain. The positions of E1p, E2p (1lip-8lip), E3, and GroEL (a frequent contaminant), as well as the molecular mass markers are indicated.

amplified wild-type and $+/+$ multilip-PDH complexes (Russell *et al.*, 1992; Machado *et al.*, 1992). The subunit stoichiometries obtained by densitometric analysis of samples fractionated by SDS-PAGE (Fig. 4) varied within the range 0.5–0.7:1.0:0.9–2.0 (E1p:E2p:E3), and the specific activities ranged from 94 to 14% of those observed for 1lip-PDH complex expressed from the receptor plasmid (Table 1). Despite the presence of up to seven unlipoylated domains in the $\pm$ complexes, the 24 lipoyl cofactors on the outermost domains can clearly reach all three types of active site in the catalytic heart of the complex and support at least 14% of normal activity. The specific activities of the $\pm$ complexes are compared with those obtained previously for the $+/+$ series (Machado *et al.*, 1992) in Fig. 5(a). Those of the $\pm$ series decline gradually once the number of lipoyl domains per E2p chains exceeds four, whereas those of the $+/+$ series containing > 3 lipoyl domains per E2p chain decline in an irregular manner, but retain at least 19% of the maximum observed activity.

It had been thought that under-lipoylation might contribute to the lower activities of over-produced $+/+$ complexes, because over-expressing an independent lipoyl domain sub-gene outstrips the cell's lipoylation capacity and leads to the accumulation of unlipoylated domains (Miles & Guest, 1987; Ali & Guest, 1990). However, this does not apply to the over-produced 1lip-PDH complex (Russell *et al.*, 1992) nor should it apply to the $\pm$ series, where the number of lipoylatable domains (24) remains the same in all complexes. Attempts to increase the specific activities of the $+/+$ series by treating purified complexes with lipoate protein

Fig. 5. Comparisons of the enzymological and physiological properties of $+/+$ (■) and $\pm$ (□) series of multilip-PDH complexes. (a) Specific activities of purified PDH complexes (means of three independent preparations deviating no more than 15% from the mean): $+/+$ data plotted from Machado *et al.* (1992) and $\pm$ data from Table 1. (b) Maximum specific growth rates (μ_{max}) of isogenic derivatives of W3110 growing on glucose minimal medium in batch culture: mean values for at least three independent cultures deviating by no more than 5% from the mean.

ligase under conditions that are known to lipoylate apo-domains, were unsuccessful (data not shown). Thus it would appear that the lower specific activities of complexes containing superfluous lipoyl domains is probably due to steric interference by domains of either type (+ or -) that are not participating in the catalytic process.

The M_r values deduced from the electrophoretic mobilities of the E2p subunits were characteristically higher than those predicted from the nucleotide sequences (Table 1). The discrepancy increases as the number of lipoyl domains increases, consistent with the view that it is due to the anomalous electrophoretic behaviour of the alanine- plus proline-rich linkers (Stephens *et al.*, 1983b; Guest *et al.*, 1985; Machado *et al.*, 1992). The mean discrepancy is approximately 4000 per lipoyl domain, but the incremental changes are not uniform (Table 1). In particular, the increments associated with the addition of domains 1, 4 and 7 appeared relatively high. Such non-uniformity was also observed with the $+/+$ complexes (Machado *et al.*, 1992). The underlying reason is not known but it could be due to some form of interactive domain clustering that occurs at specific domain contents.

Construction and physiological properties of isogenic strains containing *aceF* genes that encode multiple wild-type and mutant lipoyl domains

Previous studies have shown that the physiological behaviour of growing bacterial cultures can serve as a very sensitive indicator of the performance of genetically modified PDH complexes (Davé *et al.*, 1995). Indeed, such diagnostic information is obtained under conditions which avoid variations in subunit stoichiometry or differential inactivation that may occur during enzyme purification for *in vitro* comparisons. Two sets of isogenic derivatives of *E. coli* W3110 containing altered *aceF* genes with multiple copies of the wild-type *lip* region (+/+ series) or one wild-type and up to seven mutant *lip* regions (+/- series), were accordingly constructed for physiological studies. The +/+ strains were obtained as outlined in Fig. 3, by an allelic replacement procedure that involves the co-integration of derivatives of a thermo-sensitive replicon (pMAK705; Hamilton *et al.*, 1989) and JRG2930 (*aceF::kan^R*), as used previously for lowering the *lip* content of the chromosomal *aceF* gene (Davé *et al.*, 1995). The +/- strains were obtained by transforming an *aceF::kan^R*-derivative of JC7623 (*recBC sbcBC*) with the corresponding *pdh* expression plasmids according to Oden *et al.* (1990), see Methods. Both procedures involved selecting or screening for Ace⁺ products, and made the assumption that single chromosomal copies of the altered but active *pdh* operons would restore at least some degree of acetate-independence on glucose minimal medium. This assumption proved valid because the desired products were recovered at frequencies comparable to those observed for wild-type reconstructions.

The successful construction of both series of strains indicates that single chromosomal copies of *pdh* operons containing one to nine wild-type lipoyl segments, or one wild-type segment with up to seven sub-terminal mutant segments, provide sufficient acetyl-CoA to support growth on plates of unsupplemented glucose minimal medium. However, the plate tests failed to reveal phenotypic differences that become apparent during batch culture in glucose minimal medium. Quantitative growth tests in liquid medium showed that the maximum specific growth rates ($\mu_{\max}$) vary between 100 and 33% of wild-type depending on the number and type of lipoyl domains in the PDH complex (Fig. 5b). It is clear that the highest growth rates in both series are conferred by the presence of three lipoyl domains (+++ and +-+). It would also appear that extra wild-type lipoyl domains cause significantly more impairment than the same number of mutant domains (the growth rates are reproducible to within $\pm 0.5\%$). However, it is not known whether this is due to a specific effect on catalysis or to an energetic burden that may be imposed by the increased demand for lipoyl cofactors. The genetically engineered strains exhibit a useful range of growth rates extending down to 33% of wild-type. However, apart from the general impairment associated with the higher orders of lipoyl domains, the growth rates are not directly related to the specific activities of the purified

complexes. Furthermore, if it is assumed that the complexes are not seriously impaired during their isolation, the results suggest that specific activities measured in the presence of excess substrates, are not accurate indicators of the way the enzyme performs under physiological conditions.

Another striking observation is the similar growth rates exhibited by two pairs of strains from different sets, viz. the ++ and +-2*lip* pair and the +++ and +-3*lip* pair (Fig. 5b). This similarity suggests that lipoylation of the outermost (N-terminal) lipoyl domain is of prime importance for determining the catalytic efficiency of the corresponding complex. Indeed, it is possible that the superiority of the 3*lip*-PDH complex relative to those with fewer domains, may depend more on the presence of three domains than the fact that they are all lipoylated.

A particularly sensitive test for the physiological superiority of one particular complex over another is provided by pairwise competition between isogenic strains growing in glucose-limited chemostat cultures. For example, when equal numbers of wild-type bacteria (+++) and mutants containing two lipoyl domains per E2p chain (+-) are grown together, the mutant is gradually washed out, whereas in controls containing two genetically marked wild-types, both are maintained in the same proportion for over 50 generations (Davé *et al.*, 1995). In similar chemostat competition experiments between the two 3*lip* strains, JRG2933 (+++ Nal^R) and JRG3163 (+- Nal^S), both strains were maintained in the same relative proportions for 50 generations, deviating no more than $\pm 0.5\%$ from the starting ratio at any stage. A more detailed comparison of the growth kinetics of W3110 and JRG3163 in glucose-limited chemostat culture ($D\ 0.082\ h^{-1}$) further showed that both strains exhibit the same carbon efficiencies (52–53%), protein contents (69–70%), biomass concentrations (1.46–1.47 mg dry wt ml⁻¹), carbon contents (51%), and carbon balances (93–95%). These observations strongly suggest that the corresponding +++ and +-3*lip*-PDH complexes are functionally indistinguishable.

DISCUSSION

The strategy for creating multiple duplications of part of the *aceF* gene, making use of the dual replicon (pPOD6) and unique restriction sites in the duplicated region of the replaceable cassette, generates stable *pdh* operons that express active complexes both *in vivo* and *in vitro*. The operons encoding one lipoylated domain and multiple unlipoylated domains, express high-molecular-mass sedimentable complexes having subunit stoichiometries and specific activities that are not grossly different from those containing the comparable number of lipoylatable domains. The extra domains must protrude from the E2p core without hindering core assembly or its ability to bind a normal complement of E1p and E3 subunits. Nevertheless, it seems remarkable that single lipoyl cofactors bound to the most peripheral

domains of an 8lip-PDH complex can support as much as 14% of wild-type activity. The results are consistent with the view that the lipoyl cofactors approach the acetyltransferase active site from the outer surface of the core (Mattevi *et al.*, 1992), and also with an earlier observation that a PDH complex which is totally devoid of lipoyl domains can be complemented *in vivo* by independent lipoyl domains expressed *in trans* from a genetically engineered sub-gene (Russell *et al.*, 1989). It can also be concluded that incomplete lipoylation is not responsible for the lower catalytic activities of genetically amplified PDH complexes having > 24 lipoyl-binding sites per complex. A major factor seems to be interference from superfluous domains that are not participating in catalysis. This probably accounts for the observed 50% reduction in catalytic activity of a PDH complex having a --+ configuration compared with those with having -+ and +- and +++ configurations (Allen *et al.*, 1989).

The discrepancy between the high specific activity of the +- 2lip-PDH complex and the sub-optimal growth rate of the corresponding strain, parallels the discrepancies observed previously with the + and ++ constructs (Davé *et al.*, 1995). Presumably the catalytic debility imposed by lowering the lipoyl domain content is subtle and detectable only under substrate-limiting conditions. Attempts to detect significant kinetic differences under such conditions are frustrated by the inability to isolate (or reassemble) purified PDH complexes of each type having reproducibly uniform overall specific activities, subunit stoichiometries and degrees of lipoylation. Indeed, the most sensitive indicator of catalytic competence would at present seem to be the specific growth rate of the corresponding isogenic strain.

The present results shed new light on the reason why the PDH complex of *E. coli* retains three lipoyl domains per E2p chain. Because the inner domains need not be lipoylated in order for the 3lip-PDH complex to confer the highest growth rate in glucose minimal medium, it could be argued that domain duplication both extends the reach and improves the conformational mobility of the most peripheral lipoyl cofactors. This in turn could have the effect of facilitating optimal interactions with the three types of active sites in the assembled complex, rather than simply providing extra lipoyl cofactors that can participate in catalysis. The fact that the extra cofactors do participate in catalysis may be entirely fortuitous. The view that linker reach and quality are important is supported by the significant increase (or decrease) in specific activity that accompanies the substitution of a sub-optimal polyalanine-proline linker by a polyproline (or polyalanine) linker having the same number of residues (Turner *et al.*, 1993). From an evolutionary standpoint, it would seem expedient to duplicate existing sequences rather than to improve the quality of the linker sequences by an incremental series of single Ala → Pro substitutions. On their own, such single substitutions would probably afford no significant selective advantage and, by increasing the G + C content of the gene, might even be detrimental in the long term.

If the extra lipoyl domains are primarily fulfilling a linker function (i.e. as a spacer) there should be some reason why they are still lipoylated. Possible reasons are that they: (i) confer a selective advantage under some physiological condition not tested here; (ii) are retained by intragenic recombination with the essential wild-type lip segment; or (iii) serve as a reservoir for lipoyl cofactors. It will be interesting to study the +- - strain over prolonged periods to see whether any +- +, ++ -, or +++ revertants/recombinants arise, and to set up pairwise competition experiments to find conditions in which the possession of a lipoylatable (+) rather than an unlipoylatable (-) spacer might confer a selective advantage.

The 16 isogenic strains containing wild-type or genetically engineered *pdh* operons now offer for specific purposes a choice of strains whose growth rates and carbon fluxes through the PDH complex are endogenously pre-set over a wide range (33–100% of wild-type). The strains were stable in the cultures studied here but a major factor affecting their usefulness might be their long-term stability. It will in the future be interesting to examine the effects of prolonged cultivation under glucose-limiting conditions with strains containing < 3 or > 3 lipoyl domains per E2p chain, to see whether 3lip 'revertants' are selected by internal gene duplications or deletions. The present set of engineered strains, and the strain containing an exogenously regulated *P_{tac}-aceEF-lpdA* operon, provide opportunities for redirecting metabolic carbon to commercially important products and for off-setting the deleterious accumulation of acetate.

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