Overexpression of restructured pyruvate dehydrogenase complexes and site-directed mutagenesis of a potential active-site histidine residue

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The *aceEF-lpd* operon of *Escherichia coli* encodes the pyruvate dehydrogenase (E1p), dihydrolipoamide acetyltransferase (E2p) and dihydrolipoamide dehydrogenase (E3) components of the pyruvate dehydrogenase multienzyme complex (PDH complex). A thermoinducible expression system was developed to amplify a variety of genetically restructured PDH complexes, including those containing three, two, one and no lipoyl domains per E2p chain. Although large quantities of the corresponding complexes were produced, they had only 20–50 % of the predicted specific activities. The activities of the E1p components were diminished to the same extent, and this could account for the shortfall in overall complex activity. Thermoinduction was used to express a mutant PDH complex in which the putative active-site histidine residue of the E2p component (His-602) was replaced by cysteine in the H602C E2p component. This substitution abolished dihydrolipoamide acetyltransferase activity of the complex without affecting other E2p functions. The results support the view that His-602 is an active-site residue. The inactivation could mean that the histidine residue performs an essential role in the acetyltransferase reaction mechanism, or that the reaction is blocked by an irreversible modification of the cysteine substituent. Complementation was observed between the H602C PDH complex and a complex that is totally deficient in lipoyl domains, both *in vitro*, by the restoration of overall complex activity in mixed extracts, and *in vivo*, from the nutritional independence of strains that co-express the two complexes from different plasmids.

INTRODUCTION

The pyruvate dehydrogenase (PDH) complex of *Escherichia coli* is composed of multiple copies of three enzymic components: pyruvate dehydrogenase (E1p, EC 1.2.4.1), dihydrolipoamide acetyltransferase (E2p, EC 2.3.1.12) and dihydrolipoamide dehydrogenase (E3, EC 1.8.1.4). Together they catalyse the oxidative decarboxylation of pyruvate:

The E1p, E2p and E3 components of the PDH complex are encoded by the respective *aceE*, *aceF* and *lpd* genes, which form the *aceEF-lpd* operon located at 2.8 min in the *E. coli* K12 linkage map, and at approx. 130 kb in the physical map reported by Kohara *et al.* (1987). The nucleotide sequence of an 8 kb segment carrying all three genes has been determined, allowing the amino acid sequences of the three PDH complex components to be deduced (Stephens *et al.*, 1983a,b,c). The *aceEF-lpd* operon contains two promoters: P_{ace} , which transcribes the entire operon, and P_{lpd} , which transcribes the *lpd* gene and is co-regulated with the unlinked *suc* operon in order to meet the E3 requirements of the analogous 2-oxoglutarate dehydrogenase complex. A disappointing feature of the PDH complex is the relatively poor amplification found when the genes are cloned in multicopy plasmids. This may indicate that a positive regulator is required for *ace* expression.

Research on the PDH complex has concentrated mainly on the E2p component, which has a central role in both the catalytic activity and the assembly of the complex (Reed *et al.*, 1975; Miles & Guest, 1987a; Perham *et al.*, 1987; Guest *et al.*, 1989). The E1p and E3 components are assembled on a cubic core comprising 24

E2p subunits: 12 E1p dimers at the edges of the cube, and at least six E3 dimers on the faces. The E2p component also has multiple roles in catalysis. It contains a covalently bound lipoyl cofactor that is reductively acetylated by E1p, the acetyl group then being transferred reversibly to CoA at the E2p active site. After acetyl transfer, the reduced lipoamide is reoxidized by E3 to complete the catalytic cycle and to facilitate the initiation of subsequent cycles. Thus the E2p polypeptide carries determinants for the assembly of the E2p core, the specific binding of the E1p and E3 components, the covalent attachment of lipoic acid, and the acetyltransferase active site. Biochemical studies have confirmed predictions from the DNA-derived primary structure that the E2p subunit is highly segmented, consisting of at least five independent domains connected by conformationally mobile linker sequences of 10–32 residues rich in alanine and proline (Stephens *et al.*, 1983b; Spencer *et al.*, 1984; Packman *et al.*, 1984; Packman & Perham, 1986). The N-terminal half of E2p comprises three virtually identical 80-residue domains, each of which carries a lipoic acid-attachment site. Internal to the lipoyl domains is a 50-residue segment involved in binding E3 component. The remaining 250 amino acid residues make up the inner-core domain, which contains the E1p-binding and E2p assembly functions as well as the acetyltransferase catalytic activity.

Comparisons have been made between the amino acid sequences of the acyltransferase (E2) subunits from the PDH, 2-oxoglutarate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complexes from a variety of organisms (e.g. Fussey *et al.*, 1988; Guest *et al.*, 1989). These showed that the greatest degree of sequence conservation is found in the C-terminal region, which includes the presumptive acyltransferase active site. Remote but significant sequence similarities and

Abbreviations used: PDH complex, pyruvate dehydrogenase multienzyme complex.

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predicted secondary-structural similarities have also been found between the inner-core domains of the E2 subunits and several chloramphenicol acetyltransferases (Guest, 1987; Guest *et al.*, 1989), where mechanistic and structural data are available (Kleanthous & Shaw, 1984; Leslie *et al.*, 1988). One of the most highly conserved regions includes the active-site histidine residue of chloramphenicol acetyltransferase (His-195; Kleanthous *et al.*, 1985), and it has been suggested that the equivalent residue in E2p, namely His-602, may likewise function as a general base in catalysing the acyl-transfer reaction.

In the present paper a method for amplifying the synthesis of a variety of restructured PDH complexes is described, together with an investigation of the importance of the putative active-site residue (His-602) of the acetyltransferase (E2p) component by site-directed mutagenesis of the *aceF* gene.

METHODS AND MATERIALS

E. coli strains, bacteriophages and plasmids

The following *E. coli* strains were used as hosts for M13 and plasmid derivatives: TG1 (Δ lac-*proAB supE thi hsd* Δ 5/*F'**traD36 proA⁺B⁺ lacI^qZAM15*), for routine M13 and plasmid DNA preparation and transformation; BW313 (*dut ung thiI relA spoT1/F'lysA*), for the preparation of uracil-containing template DNA for mutagenesis (Kunkel, 1985); BMH71-18mutL (Δ lac-*proAB supE thi mutL::Tn10/F' traD36 proA⁺B⁺ lacI^qZAM15*), used to increase the recovery of mutant bacteriophages (Kramer *et al.*, 1984); JRG590 (*thi relA* Δ *nadC-aceF10*), formerly H Δ 10 (Langley & Guest, 1978), used as the host strain for the expression of plasmid-encoded PDH complexes; JRG1342 (Δ aro-*lpd18 recA1 metB1 met-105 azi pox pps-1 relA rpsL*), used in growth tests to check the Ace and Lpd phenotypes conferred by plasmids (Guest *et al.*, 1985).

A derivative of M13mp18, 18LPDA (Allison *et al.*, 1988), which carries a 1.98 kb *Hind*III-*Xho*II fragment of the *aceEF-lpd* operon encoding the C-terminal half of E2p and an N-terminal segment of E3, was used for mutagenesis of the *aceF* catalytic-core-encoding region.

Plasmid pGS238 (Fig. 1; Allison *et al.*, 1988), which carries a 1.03 kb *Xho*II-SalI fragment encoding the C-terminal segment of E3, was used as an intermediate in the subcloning of mutated 18LPDA DNA, to reconstruct the *lpd* gene and ultimately the mutated *aceEF-lpd* operon.

The expression vector pJLA502 (Schauder *et al.*, 1987), which uses tandem λ P_LP_R promoters, regulated by the thermolabile *cI*857-encoded repressor, and the efficient *atpE* translation initiation region to increase the expression of cloned genes, was kindly provided by Dr. J. E. G. McCarthy, GBF-Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany.

Plasmids pGS87, pGS108, pGS185 and pGS179 (Guest *et al.*, 1985; Angier *et al.*, 1987; Miles *et al.*, 1988) encode PDH complexes whose E2p components have three, two, one and no lipoyl domains respectively (Fig. 1a). They were used to produce derivatives of pGS269, the 1-lip PDH complex expression plasmid (Guest *et al.*, 1989), which overexpress 3-, 2-, 1- and 0-lip PDH complexes.

Media

The nutritional phenotypes of derivatives of the PDH deletion strains JRG590 (Δ *nadC-aroP-aceEF*) and JRG1342 (Δ aro-*aceEF-lpd*) carrying plasmids encoding wild-type and mutant PDH complexes were determined by using minimal medium E of Vogel & Bonner (1956) with glucose (0.2%) as carbon source and supplemented where necessary with ampicillin (50 μ g/ml), thiamin (10 μ g/ml), nicotinic acid (5 μ g/ml), meth-

ionine (20 μ g/ml), succinate (2 mM) and acetate (2 mM). L broth (Lennox, 1955) with glucose (0.1%) and ampicillin (100 μ g/ml) was used as the rich medium for plasmid-carrying strains, and 2TY medium (Maniatis *et al.*, 1982) was used for growth of strains carrying bacteriophages.

Recombinant DNA techniques

Standard methods were used for M13 bacteriophage and plasmid DNA preparation, restriction-enzyme digestion, isolation of DNA fragments, ligation, transformation and transfection (Maniatis *et al.*, 1982; Glover, 1985).

Oligonucleotide-directed mutagenesis

Mutagenesis of uracil-containing 18LPDA template DNA was performed by primer extension and ligation from the mutagenic oligonucleotide according to the method of Kunkel (1985). The mutagenic oligonucleotide S83 (5'-CTCCTTCGACTgCCGCGT-GAT-3') is identical with bases 5580-5600 of the published *aceF* sequence (Stephens *et al.*, 1983b), except for mismatches at two positions (in lower case) designed to direct the desired amino acid substitution (His-602 $\rightarrow$ Cys). Mutant bacteriophages were enriched by transfection of the *ung⁺* strain BMH71-18mutL followed by plaque formation on a lawn of TG1 cells in order to limit exposure of the bacteriophage to the mutator strain. M13 DNA templates, prepared from well-separated plaques, were sequenced by the dideoxy chain-termination method with [α -³²S]thio[dATP], modified T7 DNA polymerase and buffer-gradient gels (Sanger *et al.*, 1980; Biggin *et al.*, 1983). Several clones that carried the S83 mutation were sequenced throughout the DNA segment to be subcloned, with the aid of a series of primers complementary to the 18LPDA sequence. One isolate with the expected sequence (18LPDA83) was used for subcloning.

Overexpression of PDH complexes

Strain JRG590 cells carrying thermoinducible plasmids encoding wild-type or mutant PDH complexes were grown in L broth containing glucose (0.1%) and ampicillin (100 μ g/ml) at 30 °C to mid-exponential phase ($A_{650} \geq 0.5$) and then induced by shifting to 42 °C for 3-4 h. Cells were harvested by centrifugation, washed in buffer A (20 mM-potassium phosphate buffer, pH 7.8, containing 2 mM-Na₂EDTA, 1 mM-phenyl-methanesulphonyl fluoride and 1 mM-benzamidine hydrochloride) and disrupted by ultrasonic treatment in the same buffer. The extracts were cleared by centrifugation at 100 000 *g* for 30 min to remove cells and debris. The PDH complex in the cell-free extracts was partially purified by sedimentation (at 100 000 *g* for 4 h) to enrich the complex in the pellet fraction.

PDH complex and component assays

Total PDH complex activity was assayed by the method of Danson & Perham (1976). The E1p component was assayed in cell-free extracts by the pyruvate-dependent reduction of ferricyanide (Guest & Creaghan, 1974), and in purified preparations by the 2,6-dichlorophenol-indophenol-linked assay method of Packman *et al.* (1982). The acetyltransferase activity of the E2p component was assayed in reverse by measuring the formation of acetyl-lipoamide in the presence of an acetyl-CoA-generating system by the method of Willms *et al.* (1967). The E3 component was assayed by monitoring the lipoamide-dependent reduction of NAD⁺ (Danson & Perham, 1976). Activities are expressed in units, where 1 unit represents 1 μ mol of product formed or substrate transformed per min.

SDS/PAGE

SDS/PAGE for the analysis of protein was performed by the method of Laemmli (1970) with 8% resolving gels and 4%

stacking gels containing 0.1 % SDS. Tracks contained 5–10 μ g of partially purified PDH complex or 20–25 μ g of protein from cell-free extracts, as determined by Lowry protein assay (Lowry *et al.*, 1951), with BSA as standard. Some gels were subjected to quantitative densitometric analysis after staining with Coomassie Brilliant Blue R250.

Materials

The [α - 35 S]thio]dATP (50 TBq/mmol) was supplied by New England Nuclear. Restriction endonucleases, DNA polymerase I (Klenow fragment) and T4 DNA ligase were supplied by Bethesda Research Laboratories, Boehringer Mannheim, Pharmacia–LKB Biotechnology or Northumbria Biologicals Ltd. Sequenase (modified T7 DNA polymerase) was obtained from the United States Biochemical Corp. BSA (fraction V), for use as a standard in the Lowry protein assay, was supplied by the Sigma Chemical Co.

RESULTS

Overexpression of PDH complexes

Construction of PDH-overexpression plasmids. Multicopy plasmids expressing the *aceEF-lpd* genes from their normal regulatory sequences produce amounts of PDH complex activity comparable with those in wild-type strains of *E. coli*, presumably as a result of strict control of the plasmid-borne genes (Guest *et al.*, 1989). Improved expression was therefore sought by constructing a plasmid in which the *aceEF-lpd* coding regions were placed immediately downstream of an independently controlled transcription and translation initiation sequence. Such a plasmid should facilitate the purification and analysis of wild-type and mutant complexes from enriched sources. The chosen vector, pJLA502, has tandem λ P_LP_R promoters regulated by the temperature-sensitive *cI857* repressor, and the efficient translation initiation region (TIR) from the *atpE* gene of *E. coli* (Schauder *et al.*, 1987). The initiation codon is located within an *Nco*I site, allowing coding regions in *Nco*I–*Bam*HI or *Nco*I–*Sal*I fragments to be positioned precisely for high expression. Since the ATG start codon of the *aceE* gene is not in an *Nco*I site, two complementary oligonucleotides (S32 and S33; Fig. 1b) were synthesized to form a linker that encodes the first ten codons of the *aceE* structural gene and has single-stranded overhangs to facilitate its ligation between the *Nco*I and *Bam*HI sites of pJLA502 to form pGS230. The presence of the linker sequence in this intermediate plasmid was checked by restriction mapping and by hybridization with end-labelled linker DNA. The vector *Nco*I site is not regenerated by insertion of the linker because of sequence constraints imposed by the second codon of *aceE* (Fig. 1b). Use of this linker ensured that the *aceE* start codon was well placed for high expression. It also allowed the remainder of the restructured 1-lip *aceEF-lpd* operon encoded by pGS110 (Guest *et al.*, 1985) to be subcloned between the unique *Bam*HI and *Sal*I sites of pGS230, by using the *Bam*HI site in the tenth codon of the *aceE* coding region, and a *Sal*I site approx. 3 kb beyond the end of the *lpd* gene (Fig. 1c). The resulting plasmid, pGS264, was checked by restriction mapping and sequencing of approx. 500 bases spanning the vector–*aceE* junction. The phenotype conferred by pGS264 was tested by growing transformants of the *ace-lpd*-deletion strain JRG1342 on minimal media. At 30 °C the transformants had an Ace[–]Lpd⁺ phenotype as a consequence of expression from the independent *lpd* promoter, but this was converted into an Ace⁺Lpd⁺ phenotype at 37 °C by thermoinduction of the λ promoters. The unwanted region distal to the *lpd* gene was removed from pGS264 by replacing the 5.7 kb *Sph*I–*Sal*I fragment with the corresponding 2.5 kb

*Sph*I–*Sal*I fragment of pGS239, which contains an engineered *Sal*I site immediately downstream of the *lpd* gene (Fig. 1c; Allison *et al.*, 1988). The resulting plasmid, pGS269, conferred an Ace⁺Lpd⁺ phenotype on strain JRG1342 at 37 °C and overexpressed the components of the 1-lip PDH complex upon thermoinduction. Plasmids that overexpressed PDH complexes with three (pGS282), two (pGS283), one (pGS284) or no (pGS285) lipoyl domains per E2p chain were produced by replacement of the *Bam*HI–*Sph*I fragment of pGS269 with the corresponding fragment from plasmids pGS87, pGS108, pGS185 or pGS179 (Figs. 1a and 1c). The creation of an isogenic set of plasmids that overexpress 3-, 2-, 1- and 0-lip PDH complexes is an extension of the cassette-based mutagenesis strategy employed throughout the investigations of PDH complex structure and function (Guest *et al.*, 1989). These plasmids were designed so that unique cloning sites could be used for substituting mutagenized segments of the *aceEF-lpd* operon, in order to overexpress a diverse range of mutant PDH complexes.

Expression of PDH complexes from thermoinducible λ promoters. The expression plasmid encoding the 1-lip PDH complex, pGS284, was used to define the conditions for optimal expression. Of the conditions tested, growth in rich medium at 30 °C and induction at 42 °C for 3–4 h gave the best expression of PDH-complex activity from these plasmids (see the Methods and materials section for details). Comparisons with the wild-type strains HfrH and W3110 under the same growth and induction regime showed that the activities of thermoinduced PDH complexes of cell-free extracts of JRG590 carrying pGS284 or pGS269 (2.0–2.5 units/mg of protein) were typically only 2–5-fold higher than the activities of the wild-type (0.5–1.0 units/mg of protein). However, analysis of the proteins in the cell-free extracts by SDS/PAGE indicated that a substantial amplification of the PDH-complex components had occurred (Fig. 2). Whereas the subunits were undetectable in wild-type extracts, a densitometric analysis of stained gels of extracts of the plasmid-carrying strains indicated that the three PDH-complex components comprised 25–30 % of the soluble protein after thermoinduction (about 15–25 mg of complex/litre of culture). It should be noted that expression of E3 was high before induction owing to the presence of the independent *lpd* promoter, and that less than 10 % of total PDH-complex protein was found in the insoluble fraction after thermoinduction.

In further studies the overexpressed PDH complexes were partially purified from cell-free extracts by sedimentation at 100 000 *g* for 4 h. Over 95 % of the PDH-complex activity was recovered in the pellet, confirming that the overexpressed components are able to assemble into high-molecular-mass complexes. The sedimented PDH complexes were estimated to be approx. 50 % pure by densitometric analysis of stained gels (Fig. 3). The corresponding PDH complex and component specific activities were measured and a typical set of results is presented in Table 1. The overall PDH-complex specific activities were 2–5-fold lower than would be predicted for 50 %-pure complexes, whereas the acetyltransferase (E2p) activities were consistent with the estimated purity. The shortfalls in PDH-complex activity correlated with low E1p activities. Indeed, the densitometric analysis of stained gels indicated that the overexpressed complexes were deficient in E1p. This probably accounts for the low specific activities of the complexes, since depletion of the rate-limiting E1p component is known to diminish overall specific activity (Bates *et al.*, 1977). The E3 activities were invariably higher than predicted, but this is not unexpected since plasmid-encoded complexes generally contain excess E3 subunits (Guest *et al.*, 1985).

Experiments with a thermoinducible expression plasmid

Fig. 1. Structure and construction of PDH-overexpression plasmids

(a) General structure of the pBR322-based plasmids encoding PDH complexes with three, two, one and no lipoyl domains per E2p chain. The number of lipoyl domains encoded by each plasmid is indicated by a superscript numeral. (b) The synthetic linker sequence used to place the *aceE* start codon in the optimal position for expression from the vector promoters. The linker carries base changes at four positions to optimize codon usage. (c) Plasmids overexpressing PDH complexes with three, two, one and no lipoyl domains per E2p chain were constructed by subcloning the appropriate fragments from the plasmids illustrated above into a pJLA502-based receptor plasmid, as described in the text. (d) The mutagenized *SphI*-*XhoII* fragment from the M13 derivative 18LPDA83 was inserted between the *SphI* and *BamHI*/*XhoII* sites of pGS238 to produce pGS316. The *SphI*-*Sall* fragment from pGS316 was subcloned into pGS284 to form pGS318, which overexpresses a 1-lip version of the H602C PDH complex. Specific features are denoted thus: thick line, pBR322 sequences; hatched lines, pJLA502 sequences; white boxes, coding regions of the *aceEF*-*lpd* operon; black boxes, synthetic linker sequences; arrows, promoters. Restriction enzyme sites are shown as follows: B, *BamHI*; H, *HindIII*; S, *SalI*; Sp, *SphI*; St, *SstI*; XI, *XhoI*; XII, *XhoII* (not unique); B/XII, *BamHI*/*XhoII* hybrid site. Square brackets indicate sites created by mutagenesis. The [St] site is only present in pGS185, pGS284 and pGS318. The position of the mutated residue in pGS316 and pGS318 is marked by an asterisk.

encoding the independent lipoyl domain from an *aceF* subgene have shown that a significant proportion of the overexpressed domain is non-lipoylated (Miles & Guest, 1987b; S. T. Ali & J. R. Guest, unpublished work). Thus it is conceivable that the E2p components of the overexpressed complexes are so poorly lipoylated that active-site coupling cannot compensate entirely for the lack of acetyl-carrying capacity. Such an effect would be masked by the deficiencies in E1p, but the observation that E2p from the overexpressed pGS284-encoded complex can never-

theless be reductively acetylated (result not shown) implies a reasonable level of lipoylation.

Although the overexpression plasmids produce only a moderate increase in PDH-complex activity, they express large amounts of easily purified PDH protein in the form of high-molecular-mass complexes with near-normal E2p and E3 activities. They may then be useful in the rapid analysis of E2p mutants, where the availability of large amounts of complex may outweigh the problems of low E1p and overall complex activity.

Fig. 2. Heat induction of *E. coli* W3110 and JRG590[pGS284]

Cultures grown at 30 °C to mid-exponential phase were induced by heating at 42 °C and sampled during a 4 h induction period. Samples of the cell-free extracts (25 µg of protein/lane) were analysed by electrophoresis in SDS/8% polyacrylamide gels and stained with Coomassie Brilliant Blue. The lane marked M contains partially purified 1-lip PDH complex, and the positions of the E1p, 1-lip E2p and E3 bands are shown.

Oligonucleotide-directed mutagenesis of a potential active-site residue

Comparisons between a number of E2 sequences and that of chloramphenicol acetyltransferase have shown a high degree of conservation in the C-terminal region and implicated His-602 of E2p as a potential active-site residue (Guest, 1987; Guest *et al.*, 1989). Site-directed mutagenesis was used to replace the corresponding histidine codon (CAC) with a cysteine codon (UGC), and the effects on both the E2p and overall complex activities were investigated. This substitution was chosen because a cysteine residue in the E2p active site might be accessible to modification by thiol-active reagents in a substrate-protectable manner, and might even be directly acetylatable by acetyl-CoA or acetyl-lipoamide.

An M13mp18 derivative carrying the appropriate segment of *aceF*, 18LPDA, was subjected to oligonucleotide-directed mutagenesis to produce the mutant bacteriophage 18LPDA83 as described above (see the Methods and materials section). The 1.5 kb *SphI*–*XhoII* fragment of 18LPDA83 that encodes the E2p catalytic region was subcloned between the *SphI* and *BamHI* sites of pGS238 to create pGS316 (Fig. 1d). The mutation was then transferred into the 1-lip PDH-complex-overexpression plasmid pGS284 on a 2.5 kb *SphI*–*SalI* fragment, making pGS318. The presence of the expected mutation in pGS318 was confirmed by restriction mapping with *DraIII*, which cleaves in the sequence CACNNCTG. One *DraIII* site in pGS284 (CACCGCCTG) is lost in the mutant plasmid pGS318 (TGCCGCCTG), which encodes a PDH complex whose E2p components have a single lipoyl domain and the amino acid substitution His-602 → Cys.

A partially purified pGS318-encoded PDH complex was prepared by ultracentrifugation and assayed for PDH-complex and component activities. This H602C PDH complex was indistinguishable from the analogous pGS284-encoded 1-lip

Fig. 3. Overexpression and purification of the restructured PDH complexes

(a) Cell-free extracts (20 µg of protein) and (b) partially purified PDH complexes (10 µg protein) from thermoinduced cultures (4 h at 42 °C) of *E. coli* JRG590 carrying the plasmids indicated were analysed by electrophoresis in SDS/8% polyacrylamide gels and stained with Coomassie Brilliant Blue. Bands corresponding to E1p, E3 and the 3-, 2-, 1- and 0-lipoyl domain forms of E2p (E2p³, E2p², E2p¹ and E2p⁰) are indicated. The position of the 61 000 Da proteolytic fragment of E1p is shown by an open triangle.

complex in its thermoinduction, its assembly into high-molecular-mass complex, and in its E1p and E3 activities (Table 1). However, the H602C PDH complex had no detectable overall complex or E2p activity, indicating that His-602 is important for the functional integrity of E2p.

Complementation between mutant PDH complexes

The H602C E2p component lacked dihydrolipoamide acetyltransferase activity, but nevertheless formed a high-molecular-mass complex binding E1p and E3 components. This indicates that the amino acid substitution that renders H602C E2p inactive does not cause substantial structural changes affecting subunit folding and assembly of the complex. A complementation test was devised to determine whether the lipoyl domain of the catalytically inactive H602C E2p component could function as a substrate in the reactions catalysed by the E1p, E2p and E3 subunits. This involved the use of the pGS285-encoded 0-lip PDH complex, which retains full dihydrolipoamide acetyltransferase activity, but has no overall complex activity owing to its lack of lipoyl domains. The mobility of the interdomain linker sequences of the E2p component allows the lipoyl domains to service many active sites in the same complex (Miles *et al.*, 1988). Thus it seemed likely that the lipoyl domains of the H602C PDH complex might carry acetyl groups to the E2p active sites of the 0-lip complex, rendering the combination of H602C and 0-lip complexes active where each complex alone had no activity. It was further envisaged that active complexes containing a mixture of H602C and 0-lip E2p subunits could be produced under conditions favouring disassembly of the individual complexes. In the same way, active hybrid complexes

Table 1. PDH-complex and component specific activities of the overexpressed PDH complexes with three, two, one and no lipoyl domains per E2p chain or a mutant E2p catalytic domain (H602C)

PDH complexes prepared by sedimentation, and estimated by densitometry to be about 50 % pure, were assayed for overall PDH-complex and individual component activities. All assays were performed in triplicate on at least two preparations of complex. Typical values for native PDH complex purified from *E. coli* are also given (S. J. Angier, unpublished work). One unit of enzyme catalyses the formation or transformation of 1 μ mol of product or substrate per min.

Plasmid	Type of E2p	Enzyme specific activities (units/mg of protein)			
		PDH complex	E1p	E2p	E3
pGS282	3-lip	2.8	0.017	3.4	64
pGS283	2-lip	3.0	0.017	3.5	34
pGS284	1-lip	6.4	0.040	3.6	59
pGS285	0-lip	< 0.003	0.087	4.7	122
pGS318	1-lip H602C	< 0.003	0.033	< 0.0007	52
<i>E. coli</i>	3-lip	25	0.1	7	50

might be formed *in vivo* by co-expressing the plasmids encoding the 0-lip and H602C complexes in the same cytoplasm.

Complementation *in vitro*. Several methods were employed in attempts to recover overall PDH complex activity from mixtures of the partially purified H602C and 0-lip complexes. In each case the effects of the treatment on PDH-complex activity was examined with a similar preparation of the pGS284-encoded 1-lip PDH complex. Significant activity (0.02 unit/mg of protein) was obtained by simply mixing the two complexes, and the degree of complementation increased with time up to about 7 h (Fig. 4). The steady increase in activity suggests that a slow exchange of subunits or subcomplexes might be occurring under non-dissociating conditions. The highest activity (0.36 unit/mg of protein) was 5.8 % of the specific activity of the partially purified overexpressed 1-lip PDH complex (Table 1). Controls in which the H602C and 0-lip complexes were incubated separately for 24 h before mixing gave 0.06 unit/mg of protein. This suggested that some dissociation of the complexes had occurred during the 24 h incubation, allowing more activity to be recovered upon mixing. Nevertheless, it was clear that an extended period of mixed incubation was essential for maximal complementation. The activity of the 1-lip complex was not affected by incubation under the same conditions.

Significant, but less extensive, complementation was observed with treatments that favour reversible disassembly of the complexes. The H602C and 0-lip complexes were mixed in the presence of 2 M-NaCl or 4 M-urea (for 1 h at 20 °C) followed by dialysis against buffer A (for 18 h at 4 °C), to facilitate the formation of hybrid complexes. The PDH-complex activities of the salt-treated and urea-treated mixtures (0.07 and 0.15 unit/mg of protein respectively) were significantly greater than the activities of comparable samples mixed after treatment (0.01 unit/mg of protein). In control experiments the activity of the partially purified pGS284-encoded 1-lip PDH complex was diminished by 66 % after salt treatment (1.0 unit/mg of protein) but was not significantly affected by the urea treatment (3.0 unit/mg of protein).

Complementation *in vivo*. Complementation was investigated *in vivo* to determine whether higher specific activities could be

Fig. 4. Complementation *in vitro* between the H602C and 0-lip PDH complexes

A mixture containing partially purified H602C and 0-lip PDH complexes (500 μ g of protein each, in 0.5 ml of buffer A) was incubated at 20 °C. The mean specific activities of duplicate hourly samples (unit/mg of total protein) are plotted versus time of incubation (O). No detectable activity (< 0.003 unit/mg of protein) was observed with either of the complexes incubated alone (●), but activity was still recovered upon mixing after 24 h (□).

achieved by allowing hybrid complexes to assemble within the cell, and whether this activity could reverse the nutritional phenotype of an *aceEF*-deletion mutant. For this purpose a pGS285 (0-lip E2p) transformant of JRG590 (Δ *nad-aceF*) was further transformed with pGS318 (1-lip, H602C E2p). Since pGS285 and pGS318 carry the same replication origin and drug-resistance, it was not possible to co-select for the presence of both plasmids in a single host bacterium with ampicillin. However, selection on glucose minimal medium supplemented with ampicillin produced a high frequency of *Ace*⁺*Ap*^r transformant colonies, each containing both pGS285 and pGS318, and capable of good growth in the absence of acetate. This indicates that PDH-complex activity can be generated *in vivo* by the assembly of hybrid (H602C + 0-lip) complexes. Cell-free extracts of cultures of JRG590[pGS285,pGS318], grown to mid-exponential phase in glucose minimal medium at 37 °C and then incubated at 42 °C for 3 h, had PDH-complex activities (1.0 unit/mg of protein) that were 40 % of those of comparable extracts of JRG590 [pGS284]. Analysis of the extracts by SDS/PAGE showed that overexpression of both plasmids had occurred in the dual transformants because the E1p, 1-lip H602C E2p, 0-lip E2p and E3 bands were clearly visible (results not shown). Control experiments in which induced cultures of JRG590[pGS285] and JRG590[pGS318] were mixed before sonication produced cell-free extracts with PDH-complex activities of 0.1–0.2 unit/mg of protein. This suggests that the hybrid H602C+0-lip PDH complexes are formed more efficiently *in vivo* than during sonication of the harvested cells.

These results confirm that, although the H602C E2p has no acetyltransferase catalytic activity, it retains the ability to form a high-molecular-mass complex, it binds E1p and E3, and its lipoyl domains can participate in accepting and donating acetyl groups at the active sites of the complex. A simple interpretation of these findings is that His-602 performs an essential role in the acetyl-transfer reaction, as originally deduced from comparisons with chloroamphenicol acetyltransferase (Guest, 1987; Miles & Guest, 1987a).

DISCUSSION

The overexpression system based on the thermoinducible

vector pJLA502 produced large amounts of PDH-complex protein (15–25 mg/l of culture), but it had only 20–50% of wild-type specific activity. The shortfall in activity appeared to be due to low E1p activity (Table 1). Furthermore, densitometric analyses of stained gels suggested that E1p is under-represented in the partially purified complexes. It was also clear that one of the persistent contaminating bands in the 100 000 *g*-sedimented complexes was a proteolytic E1p fragment of molecular mass 61 000 Da (Fig. 3; Radford *et al.*, 1987). Since PDH-complex activity varies with E1p content at E1p/E2p ratios below 1:1 (Bates *et al.*, 1977), it is possible that the observed underloading and low overall complex activities are due to degradation of the E1p subunit. The use of a thermoinducible expression system may exacerbate proteolysis of the complexes because the temperature increase induces expression of the heat-shock genes, including the *lon*-encoded ATP-dependent proteinase La, which may be involved in the degradation of abnormal polypeptides (Neidhardt *et al.*, 1984; Chin *et al.*, 1988). Thus, if the highly expressed components do not fold or assemble efficiently during thermoinduction, they may be particularly susceptible to proteolytic attack.

In mammalian PDH complex, covalent modification of E1p plays a major role in regulating the activity of the complex (for a review see Yeaman, 1989). It is conceivable that the E1p from *E. coli* may also be regulated, by covalent or allosteric modification, and this could mean that any method of overexpression may produce underactive E1p. The effects of other potential deficiencies, such as the underlipoylation of the E2p subunits that has been observed upon thermoinduction of lipoyl domain subgenes (Miles & Guest, 1987b; S. T. Ali & J. R. Guest, unpublished work), are probably masked by the effects of the E1p deficiency.

The replacement of His-602 by cysteine abolishes the acetyltransferase activity of E2p, apparently without any adverse effects on the other structural or functional properties of the subunit. This indicates that His-602 is an important active-site residue. Indeed, a sequence alignment for the catalytic domains of eight E2 subunits from a variety of sources shows that His-602 of *E. coli* E2p represents the only conserved histidine residue (G. C. Russell & J. R. Guest, unpublished work). This residue is

flanked by other conserved residues (DHR— $\frac{D}{N}$ G) in a region that closely resembles the conserved active-site region of the chloramphenicol acetyltransferases (—H—DG). By analogy with the acetylation of chloramphenicol, it has been suggested that His-602 of E2p functions as a general base in the acetyltransfer reaction (Guest, 1987; Miles & Guest, 1987a). The formation of a free electron-pair at position 3 of the histidine imidazole moiety is thought to facilitate the deprotonation of the CoA thiol group and the subsequent nucleophilic attack on the acetyl-lipoamide thioester. The mutant enzyme could therefore be inactive because the acetyl-transfer reaction cannot be initiated by the cysteine side chain. Alternatively, the enzyme could be inactive because the active site is blocked by modification of the thiol group of Cys-602. This residue could be irreversibly acetylated by acetyl-lipoamide, or it could form a disulphide with a CoA, lipoyl or protein thiol group. Initial attempts to acetylate the catalytic domain of the H602C PDH complex starting with [2-¹⁴C]pyruvate in the absence of CoA (Bleile *et al.*, 1981) have failed, suggesting that Cys-602 is either inaccessible or irreversibly modified in the purified complex. This mutant enzyme and related enzymes with substitutions in the same or neighbouring sites should be very useful in future studies on the catalytic activity of the acetyltransferase.

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