Further Studies with Lipoamide Dehydrogenase Mutants of *Escherichia coli* K12

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SUMMARY

The immunological properties of ten lipoamide dehydrogenase mutants of *Escherichia coli* were investigated with antiserum raised against purified lipoamide dehydrogenase. Seven mutants were CRM+ (cross-reacting material present) as they contained lipoamide dehydrogenase proteins exhibiting either complete or partial immunological identity with the wild-type protein. This indicates that at least seven of the mutations affect the lipoamide dehydrogenase structural gene (*lpd*). The remaining three mutants (CRM–) contained no detectable cross-reacting protein. None of the *lpd* mutations were sensitive to any of three different *amber*-suppressors. Genetic analysis by P1-transduction showed that all the *lpd* mutant sites were clustered very near the distal gene (*aceF*) of the *ace* region which specifies the dehydrogenase (*aceE*) and transacetylase (*aceF*) components of the pyruvate dehydrogenase multienzyme complex. Calculations based on the recombination frequency between an *aceF* mutant and the nearest *lpd* mutant site support the conclusion that apart from the possible presence of a regulatory element, the *aceF* and *lpd* genes are contiguous.

INTRODUCTION

The pyruvate and α -ketoglutarate dehydrogenase multienzyme complexes of *Escherichia coli* contain specific dehydrogenase (E1p, E1kg) and transacylase (E2p, E2kg) components but their lipoamide dehydrogenase components (E3) are functionally interchangeable and indistinguishable by several criteria. Studies with mutants have established the existence of two pairs of closely-linked genes *aceE* and *aceF* (at 2.3 min) and *sucA* and *sucB* (at 16.3 min) specifying the respective E1 and E2 components of the pyruvate and α -ketoglutarate complexes (Henning & Hertz, 1964; Herbert & Guest, 1969). Gene expression in both the *ace* and *suc* regions is polarized from the dehydrogenase to the transacylase genes (Henning, Dennert, Hertel & Shipp, 1966; Creaghan & Guest, 1972) and a regulatory element linked to and controlling expression of the *ace* genes has been detected (Flatgaard, Hoehn & Henning, 1971).

Recently mutants deficient in lipoamide dehydrogenase have been isolated and detailed studies with one of these mutants have indicated that *E. coli* possesses a single lipoamide dehydrogenase gene (*lpd*) specifying the E3 components of both complexes and that this gene is located very close to *aceF* at the distal end of the *ace* region (Guest & Creaghan, 1972; 1973; Guest, 1974).

This paper describes further immunological and genetic studies using a total of ten independent *lpd* mutants. The results confirm the existence of a single structural gene for lipoamide dehydrogenase and indicate that it is very close to and probably contiguous with the *aceF* gene. During the course of this work independent support for these conclusions

has come from studies with another mutant (sw28; *dhl*) which lacks lipoamide dehydrogenase activity (Alwine, Russell & Murray, 1973).

METHODS

Bacterial strains. The isolation and biochemical properties of the lipoamide dehydrogenase mutants, T3A58*lpdI* and 2, AB1325*lpd3*, XG3*lpd4*, WGAl*pd5*, H*lpd6*, 7, 8, 9 and 10, and details of the corresponding parental strains have been described previously (Guest & Creaghan, 1973). Spontaneous streptomycin resistant derivatives of *lpd* mutants were selected on nutrient agar containing streptomycin sulphate (200 µg/ml). All ten *lpd* mutations were also introduced into the WGAS (a streptomycin-resistant derivative of WGA; Herbert & Guest, 1969) background, to provide a series of WGAS/*lpd* mutants. To do this Leu⁺ transductants or exconjugants were selected from a leucine-requiring derivative of WGAS using *lpd* donors. The construction of strains T3A58*lpdInadC* (*trpE*, *trpA*, *lpdI*, *nadC*) and A10*nadC*(*PI*) (*aceFro*, *nadC*, *PI*) has been described previously (Guest, 1974). The Hfr and F-prime donors used for mapping were: H(Hfr, *thi*) from W. Hayes, B11 (Hfr, *met*) and R4 (Hfr, *met*) from R. H. Pritchard, F₂-*gal* (=F153, *gal*⁺)/W3101 (*galK*) from F. Jacob, KLF1 (=F101, *leu*⁺)/AB2463 (*leu*, *pro*, *his*, *arg*, *thi*, *str*) from J. D. Gross and KLF4 (=F104, *leu*⁺)/AB2463 (*argE*, *his*, *leu*, *thr*, *proA*, *recA*, *mtl*, *xyl*, *ara*, *galK*, *lacY*, *tsx*, *str*) from K. B. Low. Suppressibility of the *lpd* mutations was tested by conjugation with the F-prime donor AW9 (F14, *metB*⁺ *supU*)/AW1 (*metB*, *recA56*) from J. Scaife and by transduction with derivatives of the *trp* deletion strain, W3110 *trpR*, *trpA-E_{del}*, carrying *supD* and *supF* mutations.

Growth of organisms, preparation of cell-free extracts and enzyme assay. The media and methods used for the growth of organisms and the preparation of ultrasonic extracts have been described elsewhere (Guest & Creaghan, 1973). Extracts were stored at -20 °C. The activities for lipoamide dehydrogenase, the pyruvate and α-ketoglutarate dehydrogenase complexes and the α-ketoglutarate dehydrogenase component (E1kg) were measured spectrophotometrically according to Guest & Creaghan (1973). Pyruvate dehydrogenase (E1p) was assayed together with pyruvate oxidase by the method used for E1kg but with pyruvate as substrate.

Antiserum. A young New Zealand white rabbit was injected subcutaneously, in the neck and shoulders, on alternate days for 12 days with 0.5 ml of an *E. coli* B lipoamide dehydrogenase (1 mg protein/ml 0.03 M-potassium phosphate, pH 7.6, filter sterilized) mixed with an equal volume of Freund's incomplete adjuvant. After three weeks it received a similar course of 6 injections and was bled 14, 17 and 20 days after the last injection. From 60 ml of serum the fraction containing antibody was concentrated threefold by precipitation with ammonium sulphate at 50 % saturation followed by dialysis versus potassium phosphate buffer (0.02 M, pH 7.4 containing 0.14 M-NaCl) and stored at -20 °C. In the standard test for antibody activity, purified lipoamide dehydrogenase (5 µg protein) was incubated for 20 min at room temperature with graded amounts of antiserum in a final volume of 0.2 ml. After centrifuging (20 min, 10000 g at 4 °C) to remove precipitated antigen-antibody complex the lipoamide dehydrogenase activity remaining in the supernatant fluid was assayed. Under these conditions 4 µl was the minimum volume of concentrated antiserum to completely inactivate 5 µg of purified lipoamide dehydrogenase.

Immunodiffusion. Ouchterlony double diffusion was conducted on microscope slides (1 × 3 in) in tris-HCl buffer (5 mM, pH 7.3) containing NaCl and sodium azide (0.8 and 0.2 %, w/v, respectively) and solidified with agarose (0.9 %, w/v; Miles-Seravac). Wells (3 mm diam and 10 µl vol.) were cut in a hexagonal pattern 8 mm distant from a similar

centre well. Several dilutions of antiserum and antigen solutions were used and the most satisfactory results were obtained with 10 μ l volumes of a tenfold dilution of concentrated antiserum in the centre well and lipoamide dehydrogenase (0.1 mg/ml) or crude ultrasonic extracts of *Escherichia coli* (10 mg protein/ml) in the lateral wells. Gels were developed at room temperature for 24 to 48 h, photographed with dark ground illumination and then washed in saline and water before drying and staining with Ponceau S. The interpretation of precipitin patterns obtained with antisera specifically directed against pure proteins has been discussed by Gasser & Gasser (1971).

Genetic methods. Conjugation experiments were performed by a cross-streak mating procedure (Herbert & Guest, 1968). This procedure was modified slightly with strain AW9 which had to be concentrated 50-fold because of its relatively weak donor activity. Transductions were performed with phage P1 (Guest, 1974) and transductants were selected on glucose minimal media containing appropriate supplements. In addition, Lpd⁺ and Ace⁺Lpd⁺ transductants were also selected on enriched acetate and enriched succinate media respectively. The distribution of non-selective markers was scored by replica-plating after purification of transductant colonies.

Materials. A sample of purified *E. coli* B lipoamide dehydrogenase was kindly provided by Dr C. H. Williams, Jun. Its specific activity was initially 600 (μ mol 3-acetyl NAD reduced/mg protein/h) when assayed by the procedure adopted in the present work.

RESULTS

Immunological studies

The antiserum raised against purified lipoamide dehydrogenase (lpdh) from *Escherichia coli* B gave single precipitin lines with this antigen and with an ultrasonic extract of wild-type *E. coli* K12 (Fig. 1, A and B). These lines were fused, indicating complete immunological identity between the lipoamide dehydrogenases of the two strains of *E. coli*. The crude extracts undoubtedly contain lpdh complexed with other components of both keto acid dehydrogenase complexes in addition to some in the uncomplexed state. However, multiple precipitin lines which might correspond to these different states of lpdh were not apparent under the conditions used for immunodiffusion. Immunodiffusion tests were conducted with extracts of each of the *lpd* mutants using purified lpdh and wild-type extract in adjacent wells. Three types of reaction were observed and these are illustrated with representative mutants in Fig. 1 (C, D and F). Several mutants, *lpd*3, 4, 5 and 9, were CRM⁺ because they gave reactions identical to the wild-type, indicating the presence of cross-reacting mutant proteins (Fig. 1 D). These included the three *lpd* mutants (4, 5 and 9) which exhibited approximately 10 % of wild-type lpdh activity (Guest & Creaghan, 1973). Another group of mutants (CRM⁻) produced no detectable reaction with the antiserum; these were *lpd*6, 8 and 10 (Fig. 1, C). With the third group, consisting of mutants *lpd*2 and 7, the precipitin lines for mutant and wild-type antigens were only partially fused and this was accompanied by spur formation (Fig. 1, F). This reaction of partial identity indicates that the mutant proteins have some but not all of the antigenic sites of the wild-type protein. The corresponding antibodies can therefore diffuse past the mutant precipitin line and react with the adjacent wild-type antigen to form the spurs. The remaining mutant, *lpd*1, was difficult to classify but probably represented an extreme example of this third category because it exhibited a very weak and diffuse line of identity between two substantial spurs. Several other strains were tested, e.g. *aceE* (A2T3), *sucA* (WGAsucA26) and a mutant H2, isolated

Fig. 1. Immunodiffusion in agarose with antisera in the centre well, and lateral wells containing: 1 μ g of lipoamide dehydrogenase purified from *E. coli* B (A); ultrasonic extract equivalent to 100 μ g protein of *E. coli* K12 WGA (B and E); *hlpd10* (C); *WGAhpd5* (D); and *hlpd7* (F).

with the *lpd* mutants and tentatively identified as an *aceEsucB* double mutant. They were all CRM+, which is consistent with their Lpd+ phenotype. Another mutant H7B, probably an *ace*, *lpd* double mutant, was CRM- and the double *amber* mutant *WGAaceE64sucA35* gave a weak CRM+ reaction which is consistent with a limited expression of its wild-type *lpd* gene (Creaghan & Guest, 1972). No cross-reaction was observed with purified pig heart lpdh (Sigma). Likewise, antibodies raised against the mammalian enzyme do not precipitate the bacterial enzyme (Hayakawa, Aikawa, Otsuka & Koike, 1967).

The effect of lpdh antibodies on the activities of the overall pyruvate and α -ketoglutarate dehydrogenase complexes, the dehydrogenase component (E1) and lpdh (E3) in an ultrasonic extract of strain WGA was also investigated (Fig. 2). As in the standard test for antibody titration, antigen-antibody complexes were removed by sedimentation because they retained enzymic activity. These titrations indicated that the lpdh activities of crude extracts and the purified enzyme were equally sensitive to the antiserum. It can then be calculated that lpdh represents approximately 0.3 to 0.4 % of the total protein in the extract. The precipitation of lpdh activity was paralleled exactly by loss of overall pyruvate complex activity, whereas inactivation of the α -ketoglutarate dehydrogenase complex preceded the disappearance of lpdh. This could be due to a failure to detect low activities of the α -ketoglutarate complex, or alternatively it could indicate that the E3 component has a lower affinity for this complex. It is interesting to note that the E1 components of both complexes also appear to be precipitated with the antiserum. This is presumably a secondary effect stemming from a primary interaction between lpdh and its antibodies and the mutual

Fig. 2. The effect of lipoamide dehydrogenase antiserum on the activities of α -keto acid dehydrogenase complexes and their components in an ultrasonic extract of *Escherichia coli* K12 strain WGA. Equivalent samples of extract containing 8.5 mg protein were incubated with antiserum in buffered saline and centrifuged according to the standard procedure (see Methods) before assaying the enzyme activities remaining in the supernatant fluid: $\blacktriangle$, lipoamide dehydrogenase (E3); $\bullet$, overall pyruvate dehydrogenase complex; $\circ$, overall α -ketoglutarate dehydrogenase complex; $\blacksquare$, pyruvate dehydrogenase (E1p) plus pyruvate oxidase; $\square$, α -ketoglutarate dehydrogenase (E1kg).

affinities of the E1 and E3 components for the core component E2, which results in co-precipitation of all components. The residual E1kg activity could represent excess synthesis of this component but it may be simply due to exclusion of this component (or E1-E2 partial complex) by the antibody. The same may be true for the E1p component, where an 'excess' of this component is known to be present in the complex (Vogel, Hoehn & Henning, 1972), but the activity could in addition be due to the flavoprotein pyruvate oxidase which also catalyses the reduction of ferricyanide in the assay used.

Genetic studies

The chromosomal location of the *lpd* gene has been indicated by a detailed investigation with one strain T3A58*lpdI* (Guest, 1974). Further studies with a total of 10 *lpd* mutants are now reported.

Conjugation. The positions of all ten *lpd* mutations were restricted to the 1 to 9 minute region of the *E. coli* linkage map by cross-streak conjugation tests using several Hfr and F-prime donors (Fig. 3). In each cross *Lpd*⁺ recombinants were selected on glucose, succinate and acetate minimal media and the donors were contra-selected nutritionally or with streptomycin. *Lpd*⁺ recombinants were obtained with H, R4 and KLF4 as donors but not with B11 or KLF1; with F₂-gal a weak positive result was observed, probably due to chromosomal mobilization. Strains containing the markers shown in Fig. 3 were used to check the characteristics of the donor strains.

Fig. 3. Segment of the linkage map of *Escherichia coli* K12 showing the positions of standard markers used in conjugation studies, the points of origins of Hfr and F-prime strains and the approximate chromosomal regions carried by the F-prime factors.

Fig. 4. Genetic map showing the likely positions of *lpd* mutant loci relative to the *aceF10* and *lpd1* sites. The map distances correspond to the frequencies of *Ace*⁺*Lpd*⁺ or *Lpd*⁺ transduction expressed as a percentage of the frequency of *Nad*⁺ transduction in the same cross when the *lpd* mutants served as donors with *aceF10nadC* and *lpd1nadC* recipients. The orientation of each mutant locus relative to *aceF10* and *lpd1* is based on the distribution of the non-selective *Nad* marker in the *Ace*⁺*Lpd*⁺ and *Lpd*⁺ transductants. Also shown are the immunological characteristics of the mutants: CRM+, cross-reacting material present and indistinguishable from wild-type; CRM(+), present but exhibiting only partial identity with wild-type; CRM-, no cross-reacting material detected.

Three-factor transductional analysis. Preliminary two-factor crosses with phage P1 lysates of the *lpd* mutants indicated that all ten *lpd* sites were linked to *leu* (cotransduction frequencies of between 8 and 25 % were obtained when limited numbers of *Leu*⁺ transductants were examined during the construction of the *WGASlpd* strains, see Methods). The results of a more detailed three-factor analysis, designed to determine the positions of the *lpd* mutations with respect to the *aceF10* and *lpd1* sites are summarized in Fig. 4. For this purpose P1-lysates prepared by two successive lytic cycles on *lpd* donors were used with recipients *A10nadC(P1)* (*nadC*, *aceF10*) and *T3A58lpd1nadC* (*nadC*, *lpd1*) in turn. The frequencies of *Ace*⁺*Lpd*⁺ or *Lpd*⁺ and *Nad*⁺ transductions were determined in each cross and the former (*Ace*⁺*Lpd*⁺ or *Lpd*⁺) were expressed as a percentage of the corresponding frequency of *Nad*⁺ transduction, which ranged from 6 to 30 transductants/10⁶ P1 particles in different experiments. These recombination frequencies (map distances) all indicated close linkage between each *lpd* marker and the standard *aceF10* and *lpd1* sites. Appropriate controls to

Fig. 5. Diagrammatic representation of the gene-protein relationships of the α -keto acid dehydrogenase complexes of *Escherichia coli*. The polarities of *ace* and *suc* in RNA transcription and possible mechanisms for *lpd* transcription (Guest, 1974) are indicated. (Each complex contains multiple copies of each subunit rather than the one shown: pdh = pyruvate dehydrogenase; kgdh = α -ketoglutarate dehydrogenase).

monitor reversion were included and these were negative. The segregation of the donor Nad^+ marker was also examined with about 60 Ace^+Lpd^+ or Lpd^+ transductants from each cross. An average of 60 % (range 46 to 74 %) were Nad^+ , indicating that all the *lpd* sites are to the right of both standard markers. Distributions of less than 18 % Nad^+ would have indicated the reverse order (Guest, 1974). The positions of the *lpd2* to *10* mutations relative to each other are based solely on recombination frequencies with *lpd1*, so the order shown in Fig. 4 is by no means established. In fact, a somewhat different order would be obtained if the *lpd* to *aceF10* distances had been used. Abortive transduction was observed in all the crosses involving the *aceF* recipient but never between *lpd1* and the other *lpd* mutants, which indicates that the ten *lpd* mutants belong to a single functional unit.

Suppression. The suppressibility of the *lpd* mutants was tested with three different *amber* suppressors which insert serine (*supD*), tyrosine (*supF*) and glutamine (*supU*) in response to the *amber* codon. For this purpose the *lpd* mutations were introduced into a streptomycin-resistant derivative of strain WGA which contains a *trpA-amber* mutation and provides an independent test for suppression. Cross-streak conjugation tests with an F14supU donor failed to produce Sup^+ recombinants on several media selective for the Lpd^+ phenotype. By contrast, tryptophan-independent recombinants were obtained with all of the WGAslpd strains. In parallel studies using *trp-deletion* strains carrying *supD* or *F* as donors, tryptophan-independent transductants (which could arise only by suppression) were selected and scored for the unselected Lpd phenotype. In no case was there any evidence for suppression of the *lpd* mutations, although control tests with WGAsucA36-amber (Creaghan & Guest, 1972) were positive. It can thus be concluded that none of the *lpd* mutations is sensitive to any of these three *amber* suppressors.

DISCUSSION

The results show that all of the ten independent mutations leading to a deficiency in lipoamide dehydrogenase activity are located in one small segment of the *E. coli* chromosome. The failure to detect abortive transduction between pairs of mutants indicates that a single functional unit is affected. Furthermore, the presence of lipoamide dehydrogenase cross-reacting material in extracts of at least six of the mutants indicates that they possess missense mutations in the lipoamide dehydrogenase structural gene, *lpd*. The results confirm and extend previous findings (Guest & Creaghan, 1972; 1973; Guest, 1974; Alwine *et al.* 1973) and show that the *lpdh* components (E₃) of the pyruvate and α -ketoglutarate dehydrogenase complexes are specified by a single gene (*lpd*) which is closely-linked to the structural genes for the other components of the pyruvate dehydrogenase complex in the following order: *aceE-aceF-lpd*. This conclusion is illustrated in Fig. 5 which summarizes the gene-protein relationships of α -keto acid dehydrogenase complexes. The organization of these genes raises interesting questions concerning their evolution and the regulation of their expression, particularly the regulation of *lpd* expression relative to the other genes. Possible mechanisms for the latter have been discussed (Guest, 1974) and they depend to a large extent on the nature of the region between *aceF* and *lpd*. The cotransduction frequency measured with *aceF10* and *lpd1*, the closest CRM+ *lpd* mutant, was 97 % and values of 3.3, 1.7, 1.9, 1.3 and 2.4 % (average 2.1 %) have been obtained for the recombination frequency between the same two sites (Guest, 1974). Using the mapping function of Wu (1966) to relate cotransduction frequencies to map distances in time units, the value of 97 % corresponds to 0.02 min (assuming the length of a P₁ transducing fragment is 2.0 min). Then, if there are approximately 3000 'average genes' each containing 1000 base pairs in the 90 min linkage map (Taylor, 1970), the *aceF10-lpd1* distance corresponds to approximately 0.67 of an 'average gene'. An alternative approach to evaluating this distance comes from the estimated recombination frequency of 4.2 % between the extremities of the tryptophan synthetase *A*-gene, which corresponds approximately to 800 base pairs (Yanofsky, Drapeau, Guest & Carlton, 1967). This would indicate for *aceF10* to *lpd1* a separation of approximately 400 base pairs or 0.4 of an 'average gene'. Since the *aceF10* site is probably some distance from the distal end of the *aceF* gene (Henning & Hertz, 1964) the actual distance between the two genes may be significantly less than estimated values. These considerations lead therefore to the conclusion that the *aceF* and *lpd* genes are contiguous or separated by a relatively short regulatory region but not by any other gene(s).

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