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Mobility in pyruvate dehydrogenase complexes with multiple lipoyl domains

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High-field NMR studies were carried out with genetically-reconstructed pyruvate dehydrogenase (PDH) complexes of *Escherichia coli* containing from zero to nine lipoyl domains per lipoate acetyltransferase (E2p) subunit. The only significant differences between the NMR spectra were the increasing intensities of the signals derived from the lipoyl domains and their associated linkers, and the much enhanced signal from the E3-binding domain and its linker in complexes that are devoid of lipoyl domains. The results suggest an explanation for the presence of three lipoyl domains per E2p subunit in the wild-type PDH complex, based on its greater inherent mobility, and potentially more efficient active-site coupling, than any of the other complexes.

Pyruvate dehydrogenase complex; Lipoate acetyltransferase; Lipoyl domain; Interdomain linker peptide; Polypeptide mobility; NMR

1. INTRODUCTION

The 2-oxo acid dehydrogenase complexes contain multiple copies of three types of enzyme (E1, E2 and E3) which together catalyse the oxidative decarboxylation of 2-oxo acids to the corresponding acyl-CoA derivatives. In *Escherichia coli* the pyruvate dehydrogenase (PDH) complex contains multiple copies of the dimeric pyruvate (E1p) and lipoamide (E3) dehydrogenases assembled on the edges and faces (respectively) of a cubic core containing 24 lipoate acetyltransferase (E2p) subunits [1,2]. Each E2p subunit in turn contains five discrete domains joined by flexible linkers which are rich in alanine, proline and charged residues. There are three similar but non-identical lipoyl domains (LIP) at the N-terminal end, a small E3-binding domain (E3bd), and a large catalytic and core-forming domain at the C-terminal end.

NH_2-LIP-xxxx-LIP-xxxx-LIP-xxxxxx-E3bd-xxx-catalytic core-COOH

The linkers associated with each domain are underlined (each x denotes about five residues) and the E3bd-linker is designated as the innermost linker. The lipoyl domains bear the covalently-bound lipoyl cofactors, which are reductively acetylated, deacetylated and reoxidised at the corresponding active sites during the catalytic cycle. They are located peripherally such that their lipoyl moieties project up to 13 nm from the core to interact with the various active sites [3–5].

This paper describes work aimed at understanding why the PDH complexes of *E. coli* and other Gram-negative bacteria (e.g. *Azotobacter vinelandii*) contain three lipoyl domains per E2 subunit, whereas the E2 subunits of several related complexes contain only one or two lipoyl domains [6]. Previous studies have shown that site-directed deletion of one or two lipoyl domains has no detectable effect on the overall specific activity or active-site coupling [7]. However, PDH complex activity is abolished when all three lipoyl domains are deleted [1,8] and it declines when the number of lipoyl domains is increased from four to nine per subunit [9]. The activity is also reduced in complexes containing only one lipoyl domain per E2p subunit, if the lipoyl linker is shortened to less than half its normal length [10] or if the linker is rendered less mobile by composition changes [11]. Deletion of the innermost linker of the *A. vinelandii* PDH complex has also been shown to lower the rotational mobility of the lipoyl domain and the overall catalytic activity of the complex [12]. Here, evidence is presented to show that the three-lipoyl-domain arrangement of the wild-type PDH complex allows maximal mobility of the lipoyl linkers.

2. MATERIALS AND METHODS

2.1. *Mutagenesis, expression and purification*

Genetically restructured PDH complexes with relatively uniform subunit stoichiometries but containing from zero to nine lipoyl domains per E2p subunit [10] were overproduced and purified as de-

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scribed previously [13] except that the FPLC step was replaced by a second sedimentation step with benzamidine present but omitting PMSF.

2.2. *NMR experiments*

^{1}H NMR spectra (Fig. 1) were obtained at 500 MHz with a Bruker AMX-500 spectrometer using a spectral width of 12.5 kHz at 30°C. Data were acquired into 8,192 complex points with presaturation of the water resonance for 1.5 s. An exponential line broadening of 3 Hz was used in the Fourier transformation and chemical shifts are expressed relative to trimethyl silyl propionate. The intensities of broad signals were reduced by using spin-echo spectra with total echo times of 1 ms or 20 ms. Both conditions gave similar results but the longer delay was used because the broad envelope was more attenuated and the peaks could be integrated with greater accuracy.

Peak areas were integrated after correcting the baseline around each peak. An internal standard for PDH core intensity was taken from the composite peak at 1.7 ppm or from the aromatic envelope centred at 7.8 ppm. Both gave comparable results but the error for the aromatic envelope was greater because of its lower intensity. The intensities at 1.49 ppm, 1.4 ppm and 0.95 ppm were accordingly normalised by dividing by those at 1.7 ppm, to give intensity values for the innermost linker, the lipoyl linkers and the domains (respectively). No correction was made for other signal intensities at these positions deriving from the E1p and E3 subunits or E2p core, because their contributions cannot be established reliably. They are expected to make constant contributions to all of the observed intensities, and consequently flatten out the apparent trends in intensity values. Alternative methods for analysing the spectra gave essentially similar graphs.

3. RESULTS

Representative spectra of PDH complexes with multiple lipoyl domains are shown in Fig. 1. The absence of sharp signals is consistent with the presence of properly folded and assembled complexes. Some of the signals have been assigned previously [11,14]. The signals at 1.4

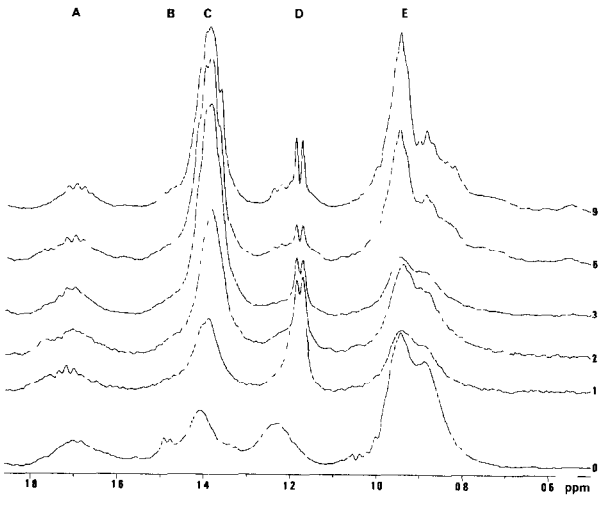
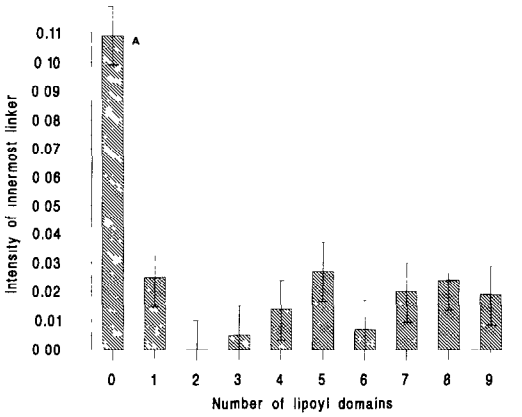
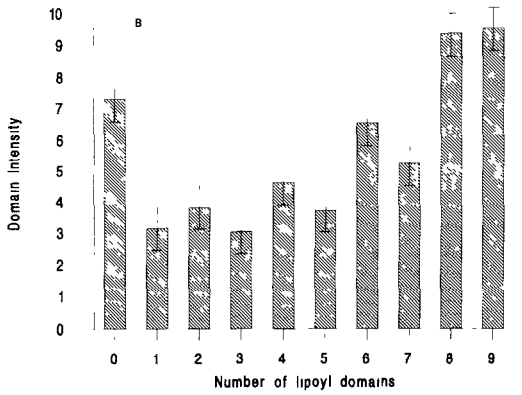
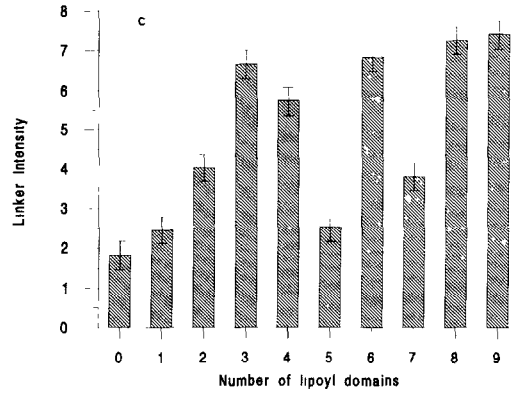


Fig. 1. ^{1}H NMR spectra of representative PDH complexes. The number of lipoyl domains per E2p subunit is indicated for each complex and the spectra have been normalised on their 1.7 ppm signals: (A) core; (B) innermost linker (E3bd-linker); (C) lipoyl linkers; (D) isopropyl alcohol (the solvent for PMSF); (E) mobile domains.







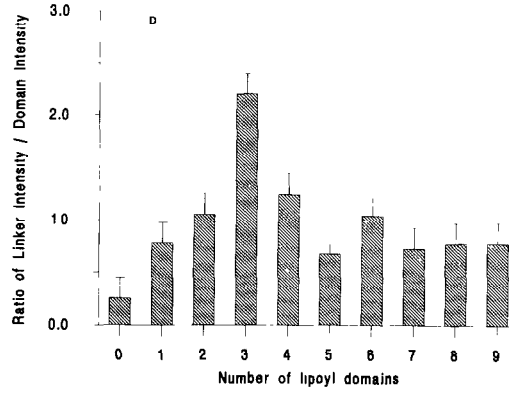


Fig. 2. Signal intensities of the PDH complexes plotted against the number of lipoyl domains per E2p subunit. Normalised spectra were used, the ordinate scales are arbitrary and the error bars denote standard deviations about the means. (A) Innermost linker intensities (1.49 ppm). (B) Domain intensities (0.95 ppm). (C) Lipoyl linker intensities (1.4 ppm). (D) Ratio of lipoyl linker intensity to domain intensity. ←

ppm derive from the methyl groups of alanine residues in the linkers associated with the lipoyl domains [14], whereas the smaller doublet at 1.49 ppm (equivalent to 1.52 ppm in [14]) comes from the alanine residues in the innermost linker (E3-bd linker). The composite signal at 0.95 ppm has been assigned to hydrophobic residues such as leucine, isoleucine and valine, found in both the E3-binding and lipoyl domains [11]. The intensities of all of these signals varied markedly with the number of lipoyl domains in the complexes. In contrast, other signals, such as the aromatic envelope and the broad signal at 1.7 ppm, showed little or no variation with the number of lipoyl domains. They are attributed to the E2p core and the E1p and E3 subunits, and they were used as internal standards for calibrating the variable signals.

The intensities of the signals at 1.49 ppm deriving from the innermost linker (E3bd-linker) are shown as a function of the number of lipoyl domains in Fig. 2A. This signal is much more intense for the PDH complex containing no lipoyl domains (0-lip complex), in agreement with previous observations showing that the innermost linker (E3-bd linker) has a high mobility only when the lipoyl domains are absent or immobile [1,14]. The intensity (and hence mobility) associated with the innermost linker remains approximately constant for all of the PDH complexes containing one or more lipoyl domains per E2p subunit.

The intensities of the signals at 0.95 ppm, largely attributed to the mobile E3-binding and lipoyl domains, are shown in Fig. 2B. The anomalously high value for the 0-lip PDH complex is due to the much higher mobility of the E3-binding domain in this complex compared with all of the others (Fig. 2A). Thereafter, the intensity increases with the number of lipoyl domains, erratically at first but accelerating later. This suggests that the outermost lipoyl domains of the 6-lip to 9-lip PDH complexes are more mobile than those situated closer to the core.

The intensities of the signals at 1.4 ppm, deriving from the lipoyl linkers, are shown in Fig. 2C, and the ratios of these intensities to the corresponding domain intensities (0.95 ppm signals) are plotted in Fig. 2D, in order to assess the mobilities of the lipoyl linkers relative to the domains. With increasing numbers of lipoyl domains, the ratio rises to a maximum for the 3-lip complex and then falls, indicating that the average relative mobility of the linkers is highest in the wild-type complex.

4. DISCUSSION

The results show that increasing the number of lipoyl domain sequences in the lipoate acetyltransferase polypeptide has no detectable effect on domain folding and complex formation, or on the structural integrity and mobility of the E2p core domains or the E1p and E3 subunits. The results also show that adding any number of lipoyl domains from one to nine lowers the mobility of the E3-binding domain by approximately the same amount, and that the outermost lipoyl domains in the 6- to 9-lip PDH complexes are mobile.

The present studies with the genetically engineered 0-lip PDH complex convincingly confirm that the innermost linker is the source of the 1.49 (1.52) ppm signals, previously assigned using trypsin-treated complexes [14], and that the E3-binding domain becomes more mobile in the absence of lipoyl domains.

The mobilities of the lipoyl linkers relative to the domains increase in going from the 1- to 3-lip complexes, as might be expected from the greater conformational freedom available to a longer polypeptide chain. More striking is the fall in relative linker mobility produced by adding further lipoyl domains to the 3-lip complex (Fig. 2D) and the fact that this is not accompanied by a corresponding fall in domain mobility (Fig. 2B). These observations could be explained by a model for the 4- to 9-lip complexes in which the outer lipoyl domains in the same or neighboring E2p subunits interact to form mobile aggregates such that the mobility of their associated linkers is restricted, but the domain aggregates remain free to move about the inner linkers.

The widely accepted model for the mechanism of the 2-oxo acid dehydrogenase complexes proposes that the lipoyl domains can pass covalently-bound intermediates between lipoyl domains in the same or neighboring subunits, channelling intermediates to unoccupied active sites and thereby increasing the efficiency of complex-mediated catalysis, a process known as active-site coupling [1,15]. According to this model, the function of multiple lipoyl domains is to increase the efficiency, and presumably the rate, of substrate transfer from one active site to another. The present work sheds light on the unexplained presence of three lipoyl domains per E2p subunit in the *E. coli* PDH complex, by showing that there is more mobility, and presumably a higher active-site-coupling efficiency, in the 3-lip complex than in the other complexes. Such a difference is not revealed by the standard assay for active-site coupling, which is an equilibrium measure, nor by specific activities determined under substrate saturating conditions, but it could be revealed by detailed kinetic measurements.

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