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Antibodies against an inter-domain segment of polypeptide chain inhibit active-site coupling in the pyruvate dehydrogenase multienzyme complex

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A synthetic peptide, AAPAAAPAKQEAAAPAPAAKAEAPAAAPAAKA, proved to be an efficient and specific immunogen in rabbits. The amino acid sequence of the peptide is identical to that of the inter-domain region (PEP3) linking the innermost of the three lipoyl domains to the dihydrolipoamide dehydrogenase-binding domain in the dihydrolipoamide acetyltransferase chain of the pyruvate dehydrogenase complex of *Escherichia coli*. Fab fragments from anti-PEP3 antibodies selectively inhibited active-site coupling in the complex without affecting the individual activities of the three component enzymes, highlighting the role of the inter-domain regions as flexible linkers in catalysis.

Pyruvate dehydrogenase; Multienzyme complex; Synthetic peptide; Antibody; Domain; Active-site coupling

1. INTRODUCTION

The pyruvate dehydrogenase (PDH) complex of *Escherichia coli* comprises an octahedral (24-subunit) core of dihydrolipoamide acetyltransferase (E2p, EC 2.3.2.12) polypeptide chains to which are bound multiple copies of the pyruvate decarboxylase (E1p, EC 1.2.4.1) and dihydrolipoamide dehydrogenase (E3, EC 1.8.1.4) subunits (review [1]). The N-terminal half of the E2p chain contains a succession of three highly homologous lipoyl domains each of about 80 amino acids [2,3]; these are linked to each other and then to a smaller (approx. 50-residue) domain responsible, at least in part, for binding the E3 subunits [4,5]. The inter-domain segments (PEP1–3) of the polypeptide chain are long (20–30-residue) sequences rich in alanine, proline and charged amino acids [2,3]. The E3-binding domain is in turn linked by a shorter and less conspicuously (alanine + proline)-rich segment to a large (approx. 29 kDa) C-terminal, inner-core domain that houses the acetyltransferase active site and binds the E1p and other E2p chains [4–6].

Movement of the lipoyl domains plays an important part in the mechanism of active-site coupling in the complex [7–9]. ^{1}H-NMR spectroscopy of the wild-type [10,11] and genetically engineered [6,12] PDH complexes and of appropriate synthetic peptides [13] has indicated that the (alanine + proline)-rich inter-domain segments of the E2p chains are indeed conformationally flexible. Two lipoyl domains can be deleted from the N-terminal end of the E2p chain without significant loss of catalytic activity in the resulting (pGS110-encoded) PDH complex [14]. However, the creation of a set of nested deletions in the (alanine + proline)-rich segment (PEP3) linking the single remaining lipoyl domain to the E3-binding domains in the pGS110-encoded E2p chain has revealed that this linker must be more than 13 residues long if it is to be fully effective in active-site coupling [15].

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We show here that a 32-residue synthetic peptide whose amino acid sequence is identical to that of PEP3 is an efficient and specific immunogen in rabbits. Fab fragments derived from antibodies directed against this inter-domain sequence do not inhibit the individual component enzymes, but their binding impairs the acetylation of lipoyl domains either by E1p directly or by intramolecular transacetylation reactions between neighbouring lipoyl domains within the E2p core. These results highlight the role of the inter-domain segments of the E2p chain in catalysis.

2. MATERIALS AND METHODS

2.1. Reagents and enzymes

Papain, phosphotransacetylase and rabbit IgG were obtained from Sigma. Peroxidase-conjugated goat anti-rabbit IgG was purchased from Toyo (Burlingame, USA). Sodium [2-^{14}C]pyruvate was a product of Amersham International. All other reagents were of analytical grade. PDH complexes were prepared from wild-type *E. coli* K12 [16] and from a suitable deletion strain of *E. coli* transformed with plasmids pGS110 [14], pGS156 [17] or pGS186-188 [15]. PDH complex activity, E1p and E3 partial activities and the acetyltransferase activity of the E2p component were measured as described [3,18].

2.2. *Preparation of antibodies and Fab fragments*

A peptide (Y-PEP3) with the sequence YAAPAAAPAK-QEAAAPAPAAKAEAPAAAPAAKA was synthesized by the solid-phase method [19]. This sequence is identical to that of the innermost long (alanine + proline)-rich region (PEP3) of the E2p chain, except for the additional tyrosine residue at the N-terminus. The tyrosine residue was used to link the peptide to bovine serum albumin and the conjugate used to immunize rabbits [20]. The IgG component was purified from the crude antiserum by ammonium sulphate precipitation followed by ion-exchange chromatography [21]. To prepare anti-PEP3 Fab fragments, IgG (20 mg/ml in 10 mM sodium phosphate buffer, pH 7.3, containing 15 mM NaCl, 2 mM EDTA and 10 mM cysteine) was incubated with papain (1%, w/v) for 3 h at 37°C and then dialysed against 10 mM sodium acetate buffer, pH 5.5, at 4°C. The insoluble (Fc) and soluble (Fab) fragments were separated by centrifugation at 12000 × *g* for 15 min at 4°C. Purification of the Fab fragments was completed by ion-exchange chromatography on a CM-cellulose column, using a linear gradient of 10 mM–1 M sodium acetate, pH 5.5 [22]. The Fab-containing fractions were pooled and dialysed vs 10 mM ammonium bicarbonate, pH 7.3, and lyophilized.

2.3. *SDS-polyacrylamide gel electrophoresis and immunoblotting*

SDS-polyacrylamide gel electrophoresis was carried out in 15% polyacrylamide slab gels and protein bands were visualised by staining with Coomassie brilliant blue followed by silver [3]. Proteins were transferred electrophoretically from SDS-polyacrylamide gels onto nitrocellulose filters [23,24] and immunoblots were developed by the method of Hawkes et al. [25] as modified by Johnson et al. [26].

2.4. *Reductive acetylation of the PDH complex and active-site coupling*

Samples of the complex [25 μg, 1 mg/ml in 20 mM sodium phosphate buffer (pH 7.0), containing 1.5 mM NAD^+ and 0.02% (w/v) NaN_3] were progressively inhibited by the addition of increasing amounts (10–40 μml) of the Fab fragments (8 mg/ml in 10 mM sodium phosphate buffer, pH 7.0). After 30 min at room temperature, a portion (2 μg) was withdrawn and the overall complex activity assayed. To each sample thiamin pyrophosphate (TPP) and $MgCl_2$ were added to give final concentrations of 0.2 and 1.0 mM, respectively, and the mixture was incubated at 37°C for 2 min. Sodium [2-^{14}C]pyruvate (1–3 μl of 23 mM, 13.1 mCi/mmol) was then added, and the extent of acetylation of the E2p core after 45 s measured as in [18]. A control experiment was performed by replacing the Fab fragments with equivalent amounts of non-specific rabbit IgG.

2.5. *Crosslinking the PDH complex with* N,N′-o-*phenylene bismaleimide*

The pGS110-encoded complex (30 μg, 1.2 mg/ml in 20 mM sodium phosphate buffer, pH 7.0, containing 1.5 mM NAD^+, 0.2 mM TPP and 1 mM $MgCl_2$) was reductively acetylated and crosslinked with *o*-phenylene bismaleimide [27]. A further sample of the complex (30 μg, 5 mg/ml in the same buffer) was also reductively acetylated as above. This sample was then incubated for 30 min at room temperature with the anti-PEP3 Fab fragments (18 μl, 15 mg/ml in 20 mM sodium phosphate buffer, pH 7.0, final enzyme concentration 1.2 mg/ml), after which the overall complex activity was assayed and found to have fallen to less than 5% of its initial value. The sample was then treated with *o*-phenylene bismaleimide [27]. A control experiment was performed by replacing the anti-PEP3 Fab fragments with Fab fragments from non-specific rabbit IgG.

3. RESULTS

3.1. *Specificity of anti-PEP3 antibodies*

The specificity of the anti-PEP3 antibodies was investigated by immunoblotting (fig.1). Only the E2p subunits of wild-type and pGS110-encoded PDH complexes were recognised by the antibodies. Little staining of any of the subunits was observed with the pre-immune serum (not shown).

To define more precisely the epitope(s) in the E2p subunits, immunoblots of the pGS156-, 186-, 187- and 188-encoded complexes were also prepared. These complexes carry a nested set of deletions in the PEP3 segment of their E2p chains [15,17]. Significantly, the antibodies failed to recognise the pGS186-, 187- and 188-encoded E2p chains (the weak staining of these bands probably arises from non-specific binding), indicating that they are highly specific for their epitopes (fig.1).

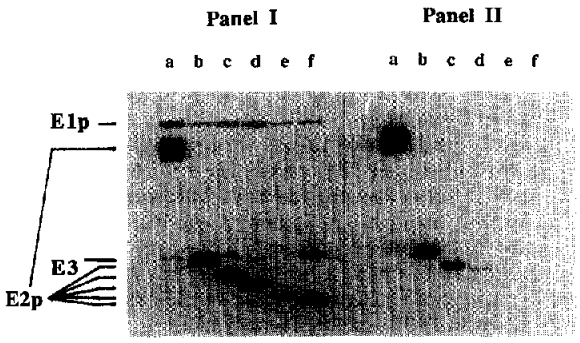

Fig.1. Specificity of anti-PEP3 antibodies. The wild-type, pGS110-, 156-, 186-, 187- and 188-encoded complexes were resolved by SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose filters and overlaid with antibodies raised against the whole complex (panel I) or anti-PEP3 antibodies (panel II). a, wild-type; b, pGS110-; c, pGS156-; d, pGS186-; e, pGS187-; f, pGS188-encoded complexes.

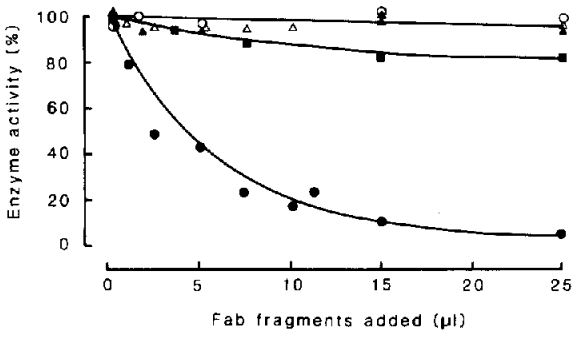

Fig.2. Effect of anti-PEP3 Fab fragments on the activity of the pGS110-encoded complex. (●) Overall complex activity; (○) E1p activity; (▲) E2p activity; (△) E3 activity; and (■) overall complex activity of control sample treated with equivalent amounts of non-specific rabbit IgG. Enzyme activities are expressed as a percentage of those of the uninhibited sample.

Since the deletions in the pGS156- and pGS186-, 187- and 188-encoded E2p chains encompass residues 2–13, 2–20, 2–26 and 2–32, respectively, of PEP3 [15,17], it is probable that one at least of the principal epitopes must lie between residues 13 and 20 of PEP3, i.e., between residues 295 and 301 [15] of the wild-type E2p chain.

3.2. *Effect of antibody binding on catalysis and active-site coupling*

To study the effect of antibody binding on the PDH complex, Fab fragments were used to avoid any crosslinking of the E2p chains by the bivalent IgG molecule. Initial experiments with the wild-type complex indicated that it was inhibited by increasing amounts of Fab fragments but that some 30% of the activity remained unaffected. The simpler pGS110-encoded complex was therefore employed for further experiments, since it retains the region PEP3 but has only one lipoyl domain per E2p chain. Samples of the pGS110-encoded PDH complex (25 µg, 0.5 mg/ml in 10 mM sodium phosphate, pH 7.0) were incubated for 30 min at room temperature with increasing amounts (0–25 µl) of the anti-PEP3 Fab fragments (16 mg/ml in the same buffer). This caused progressive and almost complete loss of the overall complex activity without effect on the activities of the three component enzymes (fig.2).

The effect of the antibodies on the intramolecular coupling of active sites [18,28,29] was therefore investigated in more detail. As the PDH complex was inhibited by the addition of increasing amounts of anti-PEP3 Fab fragments, the extent of acetylation of the E2p core with [2-^{14}C]pyruvate in the absence of CoA fell in direct proportion to the loss of overall complex activity (fig.3). In the PDH complex there normally exists a network of intramolecular transacetylation reactions, enabling acetyl groups to be passed between neighbouring lipoyl domains [28,29]. The results indicate therefore that binding of the anti-PEP3 Fab fragments to the long (alanine + proline)-rich segments of the E2p chains inhibited direct reductive acetylation of the lipoyl domains by E1p and isolated them from the network of transacetylation reactions. As a control, the addition of non-specific rabbit IgG was found to have no effect either on the overall complex activity or on the acetylation curves (not shown). Acetylation of the uninhibited complex or of a complex inhibited to about 50% of the initial enzyme activity by treatment with the anti-PEP3 Fab fragments was shown to be complete within the 45 s incubation period with sodium [2-^{14}C]pyruvate (not shown).

3.3. *Crosslinking of lipoyl domains*

The lipoyl moieties of adjacent E2p chains in the wild-type *E. coli* PDH complex can be crosslinked with *o*-phenylene bismaleimide after reductive acetylation with pyruvate [27,29]. Since the pGS110-encoded E2p chain contains only one

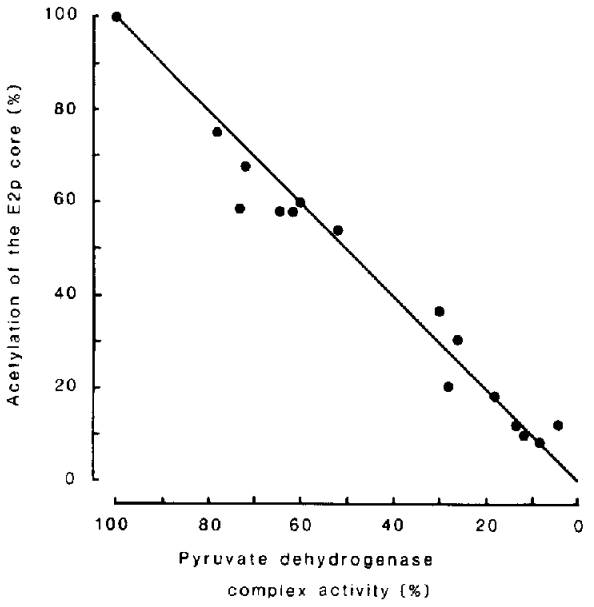

Fig.3. Reductive acetylation of the pGS110-encoded complex in the presence of anti-PEP3 Fab fragments. The overall complex activity and the proportion of E2p chains acetylated with sodium [2-^{14}C]pyruvate are expressed as a percentage of the values for the uninhibited sample.

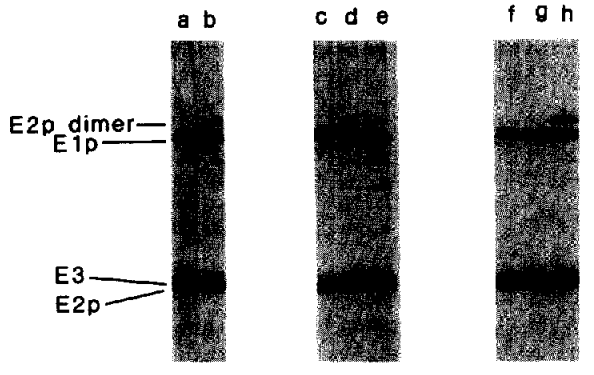

Fig.4. Crosslinking the pGS110-encoded complex with *N,N'-o*-phenylene bismaleimide in the presence of anti-PEP3 Fab fragments. The pGS110-encoded complex and complexes treated with non-specific or specific Fab fragments were exposed to *o*-phenylene bismaleimide in the presence of pyruvate. The products of crosslinking were examined by SDS-polyacrylamide gel electrophoresis, and the gel was stained with Coomassie blue followed by silver [3,27]. a, pGS110-encoded complex; b, complex treated with *o*-phenylene bismaleimide. Lanes c–e, pGS110-encoded complex inhibited with Fab fragments: c, starting material; d, sample following reductive acetylation and inhibition with Fab fragments; e as d, but complex subsequently treated with *o*-phenylene bismaleimide. Lanes f–h, as lanes c–e, but samples incubated with non-specific Fab fragments which did not recognise or inhibit the complex.

lipoyl domain, yet is fully active [14], it was expected that crosslinking in this complex would generate only E2p dimers. The pGS110-encoded complex and a complex crosslinked after exposure to non-specific Fab fragments yielded the expected E2p dimers (fig.4). However, prior binding of the anti-PEP3 Fab fragments to the pGS110-encoded complex prevented dimer formation.

4. DISCUSSION

In terms of the widely-studied problem of raising antibodies against synthetic peptides [30], it is noteworthy that peptide PEP3 was highly effective as an immunogen in rabbits. The specificity for the antigen, the long (alanine + proline)-rich region linking the innermost lipoyl domain to the E3-binding domain in the *E. coli* E2p chain, was clearly demonstrated (fig.1), and one at least of the principal epitopes appears to reside between residues 295 and 301.

The effect of the anti-PEP3 Fab fragments on the pGS110-encoded PDH complex was clear-cut and specific: inhibition of active-site coupling (fig.3) and lipoyl domain juxtaposition (fig.4) without effect on the part-reactions of the individual components (fig.2). This can be attributed to inhibition of the movements of the lipoyl domains caused by the Fab fragments binding to the segments of polypeptide chain linking the lipoyl domains to the bulk of the complex. The failure of the Fab fragments completely to inhibit the wild-type complex may be due to the additional two lipoyl domains per E2p chain being able still to participate in the reaction owing to residual flexibility in the segments of polypeptide chain linking these domains.

On the basis of their susceptibility to limited proteolysis [1,3,31] and their identification as the origin of many of the unexpectedly sharp signals detected by ^{1}H-NMR spectroscopy [10–13], the long (alanine + proline)-rich segments of the *E. coli* E2p chain are thought to be exposed to solvent and conformationally flexible. Perhaps it is not so surprising, therefore, that peptide PEP3 is strongly immunogenic and that the anti-PEP3 Fab fragments can bind effectively to the PDH complex. The lipoyl domains protrude from the inner E2p core, interdigitating between the E1p and E3 subunits (review [1]); the binding of the Fab fragments

can therefore be envisaged as the insertion of wedges into hinge regions of the E2p chains, limiting the movement of the lipoyl domains and disrupting their participation in the system of active-site coupling.

The outermost two long (alanine + proline)-rich segments of the *E. coli* E2p chain resemble in many ways the innermost one (PEP3) studied here. It will be interesting to see how these regions behave as immunogens and how this correlates with their conformations as studied by NMR spectroscopy and other biophysical techniques [13].

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