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Binding of pyruvate dehydrogenase to the core of the human pyruvate dehydrogenase complex

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Abstract In human (h) pyruvate dehydrogenase complex (PDC) the pyruvate dehydrogenase (E1) is bound to the E1-binding domain of dihydrolipoamide acetyltransferase (E2). The C-terminal surface of the E1β subunit was scanned for the negatively charged residues involved in binding with E2. βD289 of hE1 interacts with K276 of hE2 in a manner similar to the corresponding interaction in *Bacillus stearothermophilus* PDC. In contrast to bacterial E1β, the C-terminal residue of the hE1β does not participate in the binding with positively charged residues of hE2. This latter finding shows species specificity in the interaction between hE1β and hE2 in PDC.

Keywords: Subunit-binding domain; Subunit–subunit interaction; Surface plasmon resonance; PDC

1. Introduction

The pyruvate dehydrogenase complex (PDC) plays a central role in glucose metabolism by linking glycolysis and the tricarboxylic acid cycle. PDC catalyzes the oxidative decarboxylation of pyruvic acid with formation of carbon dioxide, acetyl-CoA, NADH, and H^+ through the action of the three catalytic components: (i) pyruvate dehydrogenase (E1) catalyzing the decarboxylation of pyruvate and reductive acetylation of the lipoyl moieties of dihydrolipoamide acetyltransferase (E2); (ii) E2 transferring acetyl moiety to CoA; and (iii) dihydrolipoamide dehydrogenase (E3) reoxidizing the reduced lipoyl moieties of E2 with the reduction of NAD^+ to NADH [1,2].

The structure of PDC is based on two types of symmetry: icosahedral in eukaryotes and some Gram-positive bacteria and octahedral in Gram-negative bacteria determined by the central core formed by E2. E2 has three defined structural domains connected by flexible hinge regions: (i) 1–3 lipoyl domains (two for human PDC, the outer domain, named L1, and the inner domain, named L2); (ii) E1/E3-binding (or subunit-binding) domain of E2 interacting with E1 in eukaryotes and with both E1 and E3 in bacteria; and (iii) inner domain, forming the central core of PDC and carrying the catalytic reaction of E2 [1,2]. Lipoyl groups are covalently attached to specific lysine residues of the lipoyl domains forming a "swinging arm". The lipoyl domains visit the active sites of E1, E2, and E3 coupling the individual PDC reactions and carrying acetyl moieties and reducing equivalents. Higher eukaryotes have an additional structural component, i.e. E3-binding protein (BP) with domain structure similar to E2 [3]. Human E2 (hE2) and hBP form the central core of hPDC to which all other components are bound. In mammalian PDC 20–30 heterotetramers of E1 ($\alpha_2\beta_2$) are bound to the E1-binding domain of E2 and 6–12 homodimers of E3 are bound to the E3-binding domain of BP [4].

E1s of icosahedral PDCs having tetrameric structure ($\alpha_2\beta_2$) bind to their cognate E2s through the C-terminals of their β subunits [5].The structure of the subcomplex of *Bacillus stearothermophilus* E1 (bsE1) with the E1/E3-binding domain of E2 was determined [5]. In branched-chain α-keto acid dehydrogenase complex its first catalytic component (branched-chain α-keto acid dehydrogenase) with $\alpha_2\beta_2$ structure is also suggested to bind to its cognate E2 through its β subunits close to the twofold axis [6]. E1s of octahedral PDCs which are homodimers bind to E2 through the N-terminal region as was found for *Escherichia coli* and *Azotobacter vinelandii* E1s [7,8]. Here, we identify the specific amino acids involved in the binding of hE1 to the E1-binding domain of hE2.

2. Materials and methods

2.1. Protein expression and purification

Site-directed mutagenesis was performed using Quick-change site-directed mutagenesis kit (Stratagene). The complete coding sequences of all constructs were verified by DNA sequencing. Recombinant hE1 and hE1 mutants were overexpressed in *E. coli* BL21 and purified using Ni–nitrilotriacetate–agarose chromatography as reported previously [9,10]. Recombinant hE2-BP and hE3 were overexpressed in *E. coli* and purified [9]. Recombinant hL2S [containing the second lipoyl domain (L2), second hinge region, E1-binding domain (S) and third hinge region of hE2; residues 128–330] and L2S mutants were overexpressed in *E. coli* BL21 and purified by Ni–nitrilotriacetate–agarose affinity chromatography [10]. The enzyme preparations had purities of 90–96% determined by densitometry of SDS–polyacrylamide gels (results not shown).

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Abbreviations: PDC, pyruvate dehydrogenase complex; E1, pyruvate dehydrogenase; E2, dihydrolipoamide acetyltransferase; E3, dihydrolipoamide dehydrogenase; BP, E3-binding protein; L2S, fragment containing the second lipoyl domain (L2), the second hinge region, the E1-binding domain and the third hinge region of human E2; DCPIP, 2,6-dichlorophenolindophenol; SPR, surface plasmon resonance

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2.2. Kinetic analysis and gel-filtration

Activities of wild-type and mutant hE1s were determined by two assays: (i) PDC assays, by the formation of NADH during the overall PDC reaction after reconstitution of hE1s with hE2-BP and hE3 into PDC and (ii) by 2,6-dichlorophenolindophenol (DCPIP) assays, by the reduction of DCPIP, an artificial electron acceptor, to measure the first partial reaction of E1s, the decarboxylation of pyruvate in the absence of the second substrate, lipoyl moieties of hE2 as described previously [9]. One unit of enzyme activity is defined as 1 μmol of product formed per min per mg of protein at 37 °C.

The wild-type and mutant hE1s and hL2S and its mutants were subjected to gel-filtration chromatography on Superdex HP200 to detect binding of hE1 to the E1-binding domain of hE2. E1 (200 μg) and L2S were incubated at varying ratios at room temperature for 30 min in 300 μl of 50 mM potassium phosphate buffer, pH 7.5 with 150 mM NaCl (both proteins were stable under these conditions) before applying to a Superdex HP200 column. The elution peaks corresponding to the subcomplex of two proteins and free L2S were collected and analyzed by calculating the peak areas and identification of proteins by SDS–polyacrylamide gel electrophoresis.

2.3. Surface plasmon resonance (SPR)

SPR measurements were performed on a BIAcore X instrument. hL2S was immobilized on a CM5 chip through the lipoyl moiety by a surface thiol coupling method [10]. hE1 at 0–500 nM was injected to interact with the sensor surface [10]. Duplicate runs were performed for each concentration. The reference cell on the surface of which hL2S was not immobilized was used to subtract non-specific binding. Experimental data were analyzed with BIAevaluation software to calculate the association and dissociation rate constants and the equilibrium dissociation constant.

3. Results and discussion

Prior to the structure of bsE1 bound to the E1/E3-binding domain of bsE2 was reported, the negatively charged residues and the C-terminal residues of the β subunits of bsE1 were suggested to participate in its binding to bsE2 [11]. Based on this information we screened the surface of the β subunit of hE1 for the possible candidates for electrostatic interactions and prepared eight mutants eliminating negatively charged residues: βE229A, βE229Q, βE232A, βE232Q, βE234A, βE234Q, βD289A, and βD289N (Fig. 1). Additionally we created two mutants with the C-terminal residue either replaced or deleted, i.e. βI329A and βI329del.

E1 catalyzes two successive steps: (i) the decarboxylation of pyruvate to CO_2 to form the intermediate 2-α-hydroxyethylidene-thiamin pyrophosphate and (ii) the reductive acetylation of the lipoyl groups attached to E2. Fig. 2 shows the comparison of the activities of hE1 mutants measured in two assays: DCPIP assays to determine the effect of each mutation on the first partial reaction, i.e. decarboxylation of pyruvate; and PDC assays to determine the effect of each mutation on the complete E1 reaction (two partial reactions). The first partial reaction does not require E1 to be bound to E2, while the complete E1 reaction measured by PDC assays involves interaction of E1 with E2. Mutant hE1s: βE229A, βE229Q, βE232A, βE232Q, βE234A, and βE234Q did not show drastic changes compared to the wild-type E1 activities in PDC and DCPIP assays. Activities of these mutants ranged from 67% to 106% in PDC assay and from 43% to 84% in DCPIP assay. The activities of hE1-βD289A and hE1-βD289N in the DCPIP assay did not change indicating that interactions of mutant hE1s with thiamin pyrophosphate and pyruvate were not affected. However, the hE1-βD289A mutant did not have any detectable activity in PDC assays and the activity of hE1-βD289N was reduced by 36%, suggesting that substitution with alanine prevented binding to hE2 while substitution with asparagine reduced the efficiency of interaction with hE2 but did not eliminate it completely probably by formation of a hydrogen bond instead of the salt bridge. βI329A and βI329del at the C-terminal of hE1 showed similar reductions (37–43% for βI329A and 57–62% for βI329del) in activity in both the DCPIP and PDC assays, suggesting that mutation of βI329 possibly resulted in destabilization of the protein interaction because of a local conformation change rather than by affecting the hE1 binding to hE2 directly.

Interaction of the wild-type and mutant hE1s with the di-domain (L2S) was investigated using Superdex HP200 gel-filtration analysis. Fig. 3 shows a typical elution profile of hE1 plus the L2S of hE2 at molar ratio of 1:1. The formation of a subcomplex was clearly detected (retention time 42 min). The corresponding peak of hE1-L2S subcomplex eluted before hE1 (retention time 47 min) and hL2S (retention time 52 min). By resolving the eluted protein subcomplexes formed at different molar ratios on SDS–PAGE the stoichiometry was

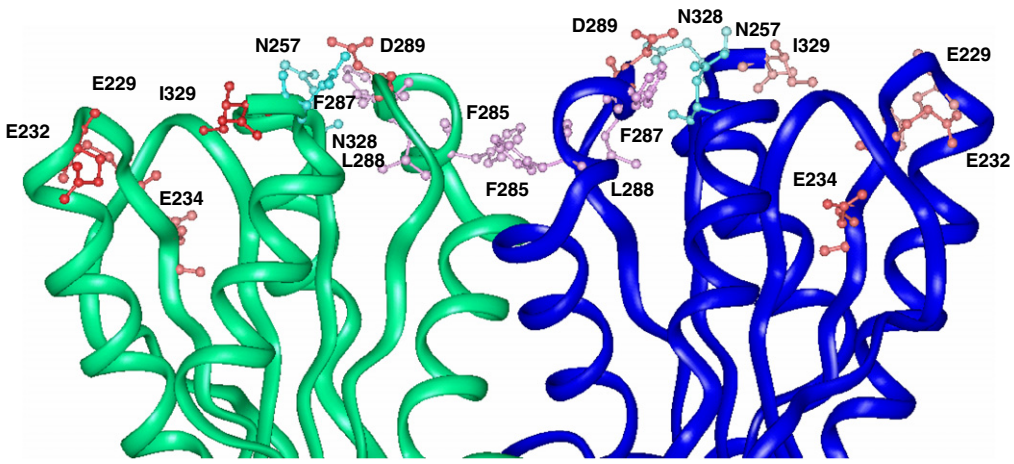

Fig. 1. The C-terminal domains of hE1β subunits. E1βs are colored green and blue. Only residues investigated and discussed in the text are displayed. The residues investigated are colored in red. The possible candidates for electrostatic interactions with hE2 are in light blue and for hydrophobic interactions with hE2 are in violet.

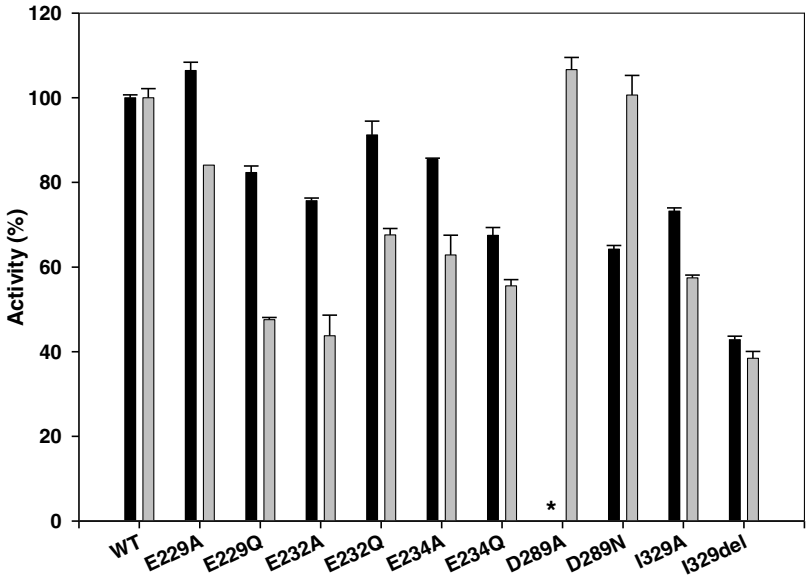

Fig. 2. Activities of the wild-type and mutant hE1s. Activities were measured in the PDC assay (black bars) by the formation of NADH after reconstitution of hE1 with hE2-BP and hE3 in PDC and by the DCPIP assay (grey bars) by the reduction of DCPIP. Results are means ± S.E. (n = 4–6). Wild-type 100% activity for E1 in PDC was 28 U/mg protein and in DCPIP-assay was 160 mU/mg protein.

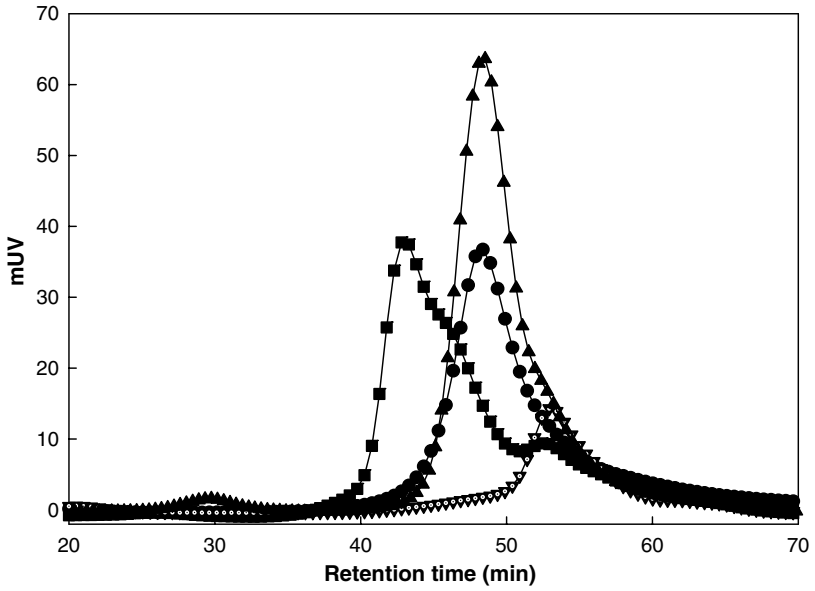

Fig. 3. Subcomplex formation between hE1-D289A and hL2S. Elution profiles of the hE1-WT and E1-D289A from Superdex HP200. (●) E1-WT (1.3 nmol); (■) E1-WT (1.3 nmol) and hL2S (1.3 nmol) at the 1:1 molar ratio; (▲) E1-D289A (1.3 nmol) and hL2S (1.3 nmol) at the 1:1 molar ratio; and (▽) L2S (1.3 nmol).

determined to be one molecule of hE1 binding to one molecule of hL2S. The same stoichiometry of binding was observed for bsPDC proteins [5,12]. The gel-filtration analysis of the hE1-βD289A interaction with hL2S revealed that a subcomplex was not formed. This finding is consistent with zero activity in PDC assay (Fig. 2).

Binding of hE1 mutants to the E1-binding domain of hE2 was directly analyzed by the SPR. hL2S was bound through the lipoyl group to the surface of the chip making it possible for the E1-binding domain of hE2 to interact with hE1. Table 1 shows the binding parameters for the wild-type and mutant hE1s. The hE1 mutants can be divided into three groups similar to the results of activity data. The mutations of residues βE229, βE232, and βE234 did not result in the large changes in the binding affinity of hE1 to hL2S. The highest fold increase in K_D is seen for βE234Q in this group, equal to only 2.9-fold. In contrast, βD289A did not show any detectable binding by SPR and the K_D for βD289N binding was about 119-fold higher compared to the wild-type hE1. Two mutants of the C-terminal of hE1 displayed only modest increases in K_D (2.5-fold for βI329A and 5.4-fold for βI329del). These changes indicate that the substitution of βI329 changes the

Table 1
Binding parameters for the wild-type and mutant hE1s interaction with the wild-type and mutant hL2Ss

E1	L2S	k_a (mM^{-1} s^{-1})	k_d (s^{-1})	K_D (nM)	Fold
WT	WT	1300	0.0123	9.47	1.0
E229A	WT	2205	0.0115	5.52	0.6
E229Q	WT	859	0.0119	13.82	1.5
E232A	WT	686	0.0121	17.8	1.9
E232Q	WT	1006	0.0120	11.85	1.3
E234A	WT	826	0.0122	14.70	1.6
E234Q	WT	601	0.0168	27.85	2.9
D289A	WT	UD	UD	UD	
D289N	WT	7.71	0.0097	1128	119
I329A	WT	822	0.0193	23.45	2.5
I329del	WT	486	0.0246	50.70	5.4
E3-WT	*WT*	*UD*	*UD*	*UD*	
WT	K276A	190.5	0.1545	810.5	86
WT	R297A	656	0.0417	64.1	6.8

Binding parameters were determined by SPR as described in Section 2.
UD, undetectable.
Italicised area shows the absence of binding between the wild-type hE3 and the wild-type hL2S.

conformation of the C-terminal of β subunits rather than that βI329 is involved in the direct interaction with the E1-binding domain of hE2. As expected hE3 was not able to bind to hL2S (Table 1).

The recently determined structure of the subcomplex of bsE1 with the E1/E3-binding domain of bsE2 [5] provides some insight into binding of hE1 to the E1-binding domain of hE2. In bacteria both E1 and E3 bind to the E1/E3-binding domain of cognate E2. Comparison of the two *B. stearothermophilus* structures of bsE1 bound to the E1/E3-binding domain of bsE2 and bsE3 bound to the same domain of bsE2 revealed that several residues of the E1/E3-binding domain of bsE2 are involved in the interactions with both bsE1 and bsE3. However, the differences in thermodynamic parameters showed that formation of the subcomplex of bsE3 with the E1/E3-binding domain of bsE2 is driven by the entropy change while a higher enthalpy change is found during the formation of the subcomplex of bsE1 with the E1/E3-binding domain of bsE2. bsE1 is bound by several electrostatic interactions, especially (residues of bsE1 and the E1-binding domain of bsE2, respectively): βE285 and E2-R136, β′F324 and E2-R157, βF324 and E2-K137; hydrophobic interactions between E2-M132 and E2-P133; the hydrophobic pocket of E1 formed by residues 281-2-86 from both β subunits; and additional hydrogen bonds including water molecules [5].

The superimposition of the hE1 and bsE1 structures (Fig. 4) revealed that hE1-βD289 occupies the same position as bsE1-βE285 and could be involved in a salt bridge with K276 of hE2 (corresponding to R136 of bsE2). This hypothesis was tested further by creating mutant K276A of hL2S. The SPR revealed that K276A had negligible binding with 86-fold higher K_D compared to wild-type hL2S (Table 1). The superimposition of the two E1 structures also uncovered that the C-terminal residues (I329) of hE1βs are located too far from either hE2-K277 (another lysine in hL2S, corresponding to K137 in bsE2) or hE2-R297 (corresponding to R157 in bsE2) to form salt bridges. The mutant R297A of hL2S was found to have K_D 6.8-fold higher than that for the wild-type hL2S (Table 1), indicating a possible involvement of this residue in the interaction; however, not with the C-terminal residue of hE1β subunit. Possible candidates for the interactions with K277 and R297 of hE2 are two residues of hE1 (βN257 and βN328) located on the surface of β subunits close to the position of the C-terminal residues of bsE1. Residues

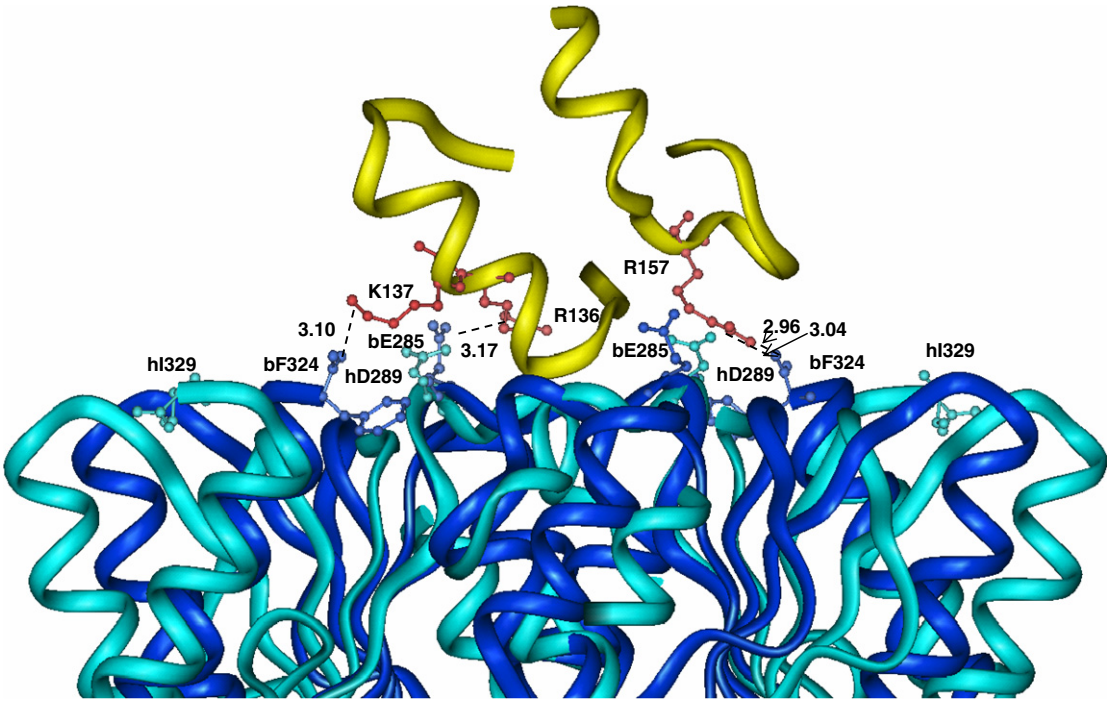

Fig. 4. Superimposition of the structures of hE1β and *B. stearothermophilus* (bs) E1β with E1/E3-binding domain of bsE2. Proteins are colored: hE1, blue; bsE1, green; the E1/E3-binding domain of bsE2, yellow (with its residues in red). Residues of interest are displayed with the side chains. Performed using InsightII/Discover software.

hE1-β284-β288 correspond to the hydrophobic pocket in bsE1 and may participate in hydrophobic interaction with hE2.

In summary, binding of hE1 involves electrostatic interactions (one of which is between hE1-βD289 and hE2-K276). The C-terminal residue of hE1β is probably not involved in the binding to hE2, and this is different from that reported for bsE1. Our findings may explain the monospecificity in the interaction between hE1 and L2S of hE2 which differs from that observed for bsE1 and bsE3 binding to the subunit-binding domain of bsE2.

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