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Interaction of E1 and E3 components with the core proteins of the human pyruvate dehydrogenase complex

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Abstract

The human (h) pyruvate dehydrogenase complex (hPDC) consists of multiple copies of several components: pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2), dihydrolipoamide dehydrogenase (E3), E3-binding protein (BP), and specific kinases and phosphatases. Mammalian PDC has a well organized structure with an icosahedral symmetry of the central E2/BP core to which the other component proteins bind non-covalently. Both hE2 and hBP consist of three well defined domains, namely the lipoyl domain, the subunit-binding domain and the inner domain, connected with flexible linkers. hE1 ($\alpha_2\beta_2$) binds to the subunit-binding domain of hE2; whereas hE3 binds to the E3-binding domain of hBP. Among several residues of the C-terminal surface of the hE1β E1βD289 was found to interact with hE2K276. The C-terminal residue I329 of the hE1β did not participate in binding to hE2. This latter finding shows specificity in the interaction between E1β and E2 in hPDC. The selective binding between hE3 and the E3-binding domain of hBP was investigated using specific mutants. E3R460G and E3340K showed significant reductions in affinity for hBP as determined by surface plasmon resonance. Both residues are involved in the structural organization of the binding site on hE3. Substitution of I157, N137 and R155 of hBP resulted in variable increases in the K_D for binding with wild-type hE3, suggesting that the binding results from several weak electrostatic bonds and hydrophobic interactions among residues of hBP with residues at the interface of dimeric hE3. These results provide insight in the mono-specificity of binding of E1 to E2 and E3 to BP in hPDC and showed the differences in the binding of peripheral components (E1 and E3) in human and bacterial PDCs.

1. Introduction

Mammalian pyruvate dehydrogenase complex (PDC) is a multienzyme complex composed of three catalytic components, namely pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2) and dihydrolipoamide dehydrogenase (E3); one structural protein called E3-binding protein (BP) and two specific families of regulatory enzymes; namely pyruvate dehydrogenase kinases (PDKs) and pyruvate dehydrogenase phosphatases (PDPs). Three catalytic components of PDC catalyze the oxidative decarboxylation of pyruvic acid with formation of carbon dioxide, acetyl-CoA, NADH and H^+. E1 carries out the decarboxylation of pyruvate and reductive acetylation of the lipoyl moieties of E2. E2 then transfers the acetyl moiety to CoA forming acetyl-CoA. E3 catalyzes reoxidation of the reduced lipoyl moieties of E2 with the reduction of NAD^+ to NADH [1;2;3]. PDC plays a key role in the maintenance

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of glucose homeostasis, and its activity is tightly regulated through the phosphorylation/dephosphorylation catalyzed by PDKs and PDPs.

PDC is a highly organized multienzyme complex. E2 and BP (in higher eukaryotes and only E2 in other organisms) form the core of the complex and bind peripheral components, i.e. E1, E3, PDKs and PDPs in higher eukaryotes [2;3]. In mammals 20–30 heterotetramers of E1 ($\alpha_2\beta_2$) are bound to the E2; 6–12 homodimers of E3 are bound to BP; 1–3 copies of PDK and 2–3 copies of PDP are bound to E2 and/or BP [4;5]. The PDC core has icosahedral symmetry in eukaryotes and some Gram-positive bacteria and octahedral in Gram-negative bacteria [4; 5]. E2 and BP have similar structures composed of three structural domains connected by flexible hinge regions: (i) the lipoyl domains [two for human PDC-E2, named L1, the outer domain and L2, the inner domain and one for human (h) PDC-BP, named L3]; (ii) the subunit-binding domain interacting with E1 and/or E3 and (iii) the inner domain, forming the central core of PDC and carrying out the catalytic reaction of E2 (Figure 1) [3]. In bacterial PDC both E1 and E3 are bound to the subunit-binding domains of E2. In higher eukaryotes E1 is bound to the subunit-binding domain of E2 and E3 to the subunit-binding domain of BP (Figure 1).

Human E1, an $\alpha_2\beta_2$ heterotetramer, has two active sites. Based on the 3D structure, the two active sites of hE1 are proposed to interact with each other during catalysis by a 'flip-flop mechanism' [6]. E1s of icosahedral PDCs having similar structures to the hE1 heterotetrameric structure ($\alpha_2\beta_2$) bind to their cognate E2s through the C-terminal of their β subunits [7]. The structure of the subcomplex of *Bacillus stearothermophilus (bs)* E1 with the E1/E3-binding domain of bsE2 was determined by Frank et al. [7]. Recently we reported several residues participating in binding of hE1 to the E1-binding domain of hE2 [8], and these findings are summarized here for comparison. E1s of octahedral PDCs which are homodimers bind to E2 through their N-terminal regions as was found for *E. coli* and *Azotobacter vinelandii* E1s [9; 10].

E3, the product of a single *Dld* gene in eukaryotes, serves as a component of three α-keto acid dehydrogenase complexes [PDC, branched-chained α-ketoacid dehydrogenase (BCKDH) complex and α-ketoglutarate dehydrogenase complex] and also as L protein in the glycine cleavage system [2]. E3 is a homodimer with two identical active sites localized at the interface between two subunits. E3 monomer has four structural domains: FAD-binding domain, NAD^+-binding domain, the central domain and the interface domain. Two tightly bound FAD molecules per dimeric E3 are involved in the electron transfer from dihydrolipoamide to NAD^+ with participation of the disulfide of the active site. In hE3 catalysis involves H452 acting as an active-site weak base, E457 stabilizing H452, and P453 positioning H452 close to the redox disulfide [11;12]. Recently the structures of hE3 with bound NAD^+ and with bound NADH [13] and as a subcomplex with the E3-binding domain of hBP were determined [14; 15]. Binding of hE3 to the E3-binding domain of hBP did not cause any detectable conformational changes in the hE3 structure. The structures indicated electrostatic as well as hydrophobic interactions between two proteins [14].

We have examined the binding regions of the hE1 to the E1-binding domain of hE2 and hE3 and the E3-binding domain of hBP. Our findings confirm the role of several amino acid residues of hBP involved in binding based on the 3D structure and provide insights in the binding of hE1 and the E1-binding domain of hE2 even though the 3D structure of this subcomplex remains unknown.

2. Experimental

2.1 Protein Expression and Purification

Site-directed mutagenesis was performed using a Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA). The complete coding sequences of all constructs were verified by DNA sequencing.

Recombinant hE1, hE1 mutants, hE2-BP, hE3, hE3 mutants, L2S [containing the second lipoyl domain (L2), second hinge region, E1-binding domain (S) and third hinge region of hE2; residues 128-330], L2S mutants, hL3S1 [containing the lipoyl domain (L3), first hinge region, E3-binding domain (S1) and second hinge region of hBP; residues 1-221] and L3S1 mutants were overexpressed and purified as reported previously [8;14;16;17]. The enzyme preparations had purities of 90–96% as judged by densitometry of SDS-polyacrylamide gels (results not shown).

2.2 Kinetic Analysis and gel-filtration

Activities of the wild-type and mutant hE1s were determined by two assays: (i) PDC assay, by the formation of NADH during overall PDC reaction after reconstitution of hE1s with hE2-BP and hE3 into PDC (to measure the complete E1 reaction, i.e. decarboxylation of pyruvate and the reductive acetylation of the lipoyl groups attached to E2).; and (ii) by DCPIP assay, by the reduction of 2,6-dichlorophenolindophenol (DCPIP), an artificial electron acceptor, to measure the first partial reaction of hE1s, the decarboxylation of pyruvate in the absence of the second substrate, lipoyl moieties of hE2 as described previously [16]. One unit of enzyme activity is defined as 1 μmol of product formed per min at 37 Â°C. Activities of free wild-type E3 and its mutants (not reconstituted in PDC) were measured in the forward reaction by the formation of NADH using dihydrolipoamide as a substrate [18].

Analysis of binding of the wild-type and mutant hE1s and L2S and its mutants by gel-filtration chromatography was performed on Superdex HP200. hE1 (200 μg) and L2S (14.3, 28.6 and 57.2 μg) were incubated at different 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 in these conditions) before applying to a Superdex HP200 column. The retention times for the elution of the complex of two proteins as well as individual unbound proteins were monitored.

2.3 Surface Plasmon Resonance

Surface plasmon resonance (SPR) experiments with hE1 and L2S were performed on BIAcore X instrument as described previouisly [8;17]. The experiments with the binding of the wild-type and mutant hE3s to the L3S1 of hBP bound to the surface of the CM5 chip were performed in the same way. L3S1 and its mutants were immobilized on the CM5 chip by a surface thiol coupling method through the lipoyl domain leaving the E3-binding domain available for E3 binding and not affected by immobilization due to a flexible hinge region between two domains. Data were analyzed with BIAevaluation software to calculate the association and dissociation rate constants and the equilibrium dissociation constant.

3. Results and Discussion

3.1 Binding of hE1 to the E1-binding domain of hE2

Frank et al. [7] demonstrated that *B. stearothermophilus* (bs) E1 is bound to its cognate E2 by electrostatic interactions and hydrophobic interactions. The main electrostatic interactions include βE285 of bsE1 with R136 of the E1-binding domain of bsE2, and interaction of two C-terminal βF324 of bsE1 with bsE2-R157 and bsE2-K137. Based on these findings of Frank et al. [7], we screened the surface of the β subunit of hE1 for negatively charged residues as

possible candidates for electrostatic interactions by investigating the following mutants: βE229A, βE229Q, βE232A, βE232Q, βE234A, βE234Q, βD289A and βD289N [8]. To determine whether C-terminal residues of the hE1β subunits are involved in binding with hE2, we investigated two mutants with substitution (βI329A) or deletion (βI329del) of the C-terminal isoleucine.

Mutant hE1s: βE229A, βE229Q, βE232A, βE232Q, βE234Q βE234A did not show any significant changes compared with the wild-type hE1 in activity in PDC and DCPIP assays indicating that residues βE229, βE232 and βE234 are not involved in binding to hE2 [8]. Figure 2 shows the comparison of the activities of hE1 mutants of βD289 and βI329 measured in two assays. hE1βD289A did not have any detectable activity in PDC assay while its activity in the decarboxylation reaction measured by DCPIP assay did not significantly change, indicating that this mutation prevented binding of the mutant hE1 to hE2. hE1βD289N also did not demonstrate any change in the DCPIP assay but its activity was reduced to 67% in PDC assay. Substitution of D289 with asparagine(N) probably reduced the efficiency of interaction with hE2, but did not eliminate it possibly through formation of a hydrogen bond instead of the salt bridge. hE1βI329A and hE1βI329del showed similar reductions in activities in both the DCPIP and PDC assays suggesting that mutations of hE1βI329 affected E1 function differently than by just inhibiting its binding to hE2 [8].

To further test the possible involvement of hE1βD289 in binding to hE2 a gel-filtration analysis of the subcomplex of the wild-type hE1 or hE1βD289A with the wild-type L2S was carried out [8]. The formation of a subcomplex formed between the wild-type hE1 and the wild-type L2S was detected at retention time 42 min while the retention time for unbound hE1 was 47 min and unbound L2S was 52 min [8]. The subcomplex of hE1βD289A and L2S was not formed (there was no peak at 42 min). Only peaks with retention times 47 and 52 min were detected corresponding to individual proteins [8].

To further investigate the binding regions of hE1 and hE2, we used a direct binding approach using SPR [8]. hL2S was bound through the lipoyl domain to the surface of the chip allowing the E1-binding domain of hE2 to interact with hE1. Figure 3 shows the x-fold increase in the K_D for binding of the mutant hE1s to the wild-type and mutant hL2Ss (relative to wild-types taken as 1 fold) [8]. As expected the mutations in hE1 of residues βE229, βE232 and βE234 did not result in large changes in the binding affinity of hE1 to L2S. hE1βD289A did not show any detectable binding by SPR, and the K_D for hE1βD289N binding was about 119-fold higher compared with the wild-type hE1 confirming the results of the kinetic and gel-filtration analyses. The two mutants of the C-terminal βI329 of hE1 displayed only moderate increases in K_D (2.5-fold for hE1βI329A and 5.4-fold for hE1βI329del). These results indicated little or no involvement of hE1βI329 in the direct interaction with the E1-binding domain of hE2.

In bsPDC the residue corresponding to βD289 of hE1 is βE285 interacting with R136 of bsE2 through a salt bridge. Superimposing the structures of hE1 (pdb 1NI4) and the complex of bsE1 with the subunit-binding domain of E2 (pdb 1W85) showed that βD289 of hE1 occupies the same position as βE285 of bsE1 and can form the salt bridge with K276 of hE2 [8]. Figure 3 shows that K_D of binding of L2SK276A to the wild-type hE1 was about 86-fold higher compared with the wild-type L2S. The K_D of another L2S mutant R297A was 6.8-fold higher than for the wild-type L2S (Fig. 3) indicating a possible involvement of this residue in the binding of hE1. The corresponding residue in bsE2, R157, participates in the salt bridge with the C-terminal residue of bsE1. The residue involved in the interaction with L2SR297 is probably different than the C-terminal residue of hE1 as was shown by superimposing the two E1 structures [8]. The C-terminal residues of hE1 are located too far from either E2K277 (corresponding to K137 in bsE2) or E2R297 (corresponding to R157 in bsE2) to form salt bridges.

Binding of hE1 to hE2 is similar to the binding in bsPDC in having one electrostatic interaction between βD289 of hE1 and K276 of hE2 but is different from bacterial PDC in the absence of an interaction of the C-terminal residue of hE1 with hE2. In contrast to hE2 which binds only E1 and does not bind E3, bacterial E1/E3-binding domain of E2 binds both E1 and E3 involving almost the same residues.

3.2 Binding of hE3 to the E3-binding domain of hBP

The structure of the subcomplex of hE3 with the E3-binding domain of hBP obtained at 2.6 Å resolution showed that the E3-binding domain of hBP binds at the interface of the two subunits of hE3 [14]. The binding involves a combination of electrostatic and hydrophobic interactions. The main residue involved in the hydrophobic interaction is I157 of hBP (interacting with Y438 of hE3), that is suggested to determine the specificity of hE3 binding to the E3-binding domain of hBP and not to hE2 (hE2 has arginine in this position). Two proline residues, P133 and P154 of hBP, also make hydrophobic contacts. Residues of hBP forming electrostatic interactions are R136 (bound to E437 of hE3), K160 (bound to D413 and E443 of hE3) and R155 (suggested to bind D444 of hE3). Several hydrogen bonds are also involved in the interactions between two proteins. The rigidity of the binding loop of the E3-binding domain of hBP with P154 (which is not present in the E1/E3 binding-domain of E2s from bacterial PDCs) and the presence of I157 in hBP instead of arginine residue in hE2 most likely determine the specificity of hE3 binding to the E3-binding domain of hBP and not to the E1-binding domain of hE2. In *B. stearothermophilus* residues R136 and R140 of the E1/E3-binding domain of E2 form electrostatic zipper interactions with E431 and D344 of E3. R136 of hBP (corresponding to conserved R136 of bsE2) is involved in the electrostatic interaction (with the same conserved residue of hE3, E437), but a positively charged residue corresponding to R140 in bsE2 is absent in hBP. Another two residues different in charge between hBP and hE2 are N137 and R155 in hBP and corresponding K277 and D295 in hE2.

We studied several mutants of hE3 prior to the structure of hE3 with the E3-binding domain of hBP was determined: K37E, K54E, P453L, E457Q, R447M, K470M, R447M/R470M, R460G and E340K. Two of these mutations were identified in E3-deficient patients: R460G and E340K [19;20]. The activity of these mutant hE3s determined in the forward E3 reaction was about: 20% for K37E, 0.5% for K54E, 34% for E457Q, 85% for R447M, 69% for K470M, 50% for R447M/K470M, 91% for R460G and 0.2% for E340K. This suggested significant roles for K54 and E340, however, this results could not provide information about the roles of these residues in binding to BP.

SPR was used to investigate further the roles of the hE3 residues in binding to hBP. The L3S1 domain was immobilized on the surface of the sensor chip and association and dissociation of hE3 were monitored (Table 1). Among the mutants investigated only two showed significant reduction in affinity for L3S1. Binding of hE3E340K to L3S1 was undetectable and the K_D for hE3R460G increased 92-fold compared to the wild-type hE3 (Fig. 4, Table 1). The other mutants did not show significant changes in binding to the E3-binding domain of hBP. L3S did not bind hE1 as expected.

After analyzing the E3-E3-binding domain of the hBP subcomplex structure (Fig. 5), it became clear that R460 of hE3 is not involved directly in the interaction with hBP; however, it is involved in the interaction (through hydrogen bonds) with residues of the neighboring subunit as well as the same hE3 subunit near the binding site for the E3-binding domain. The E3-binding domain of hBP binds at the interface between two hE3 subunits with specific residues of both the E3 subunits participating in this interaction. R460 is probably important for the structural organization of the binding site on hE3. E340 from one hE3 subunit electrostaticly interacts with R447 in the other hE3 subunit which in turn is bound through a hydrogen bond to N137 of hBP. The replacement of E340 with lysine can cause repulsion of R447 and

eliminate R447 binding to N137 of hBP. R447 of hE3 is also involved in binding to BP through N137 of hBP. R447M showed a small change in the affinity for hBP (about 3-fold increase in K_D (Table 1), probably because methionine could still participate in the interactions described above).

To investigate the role of N137, R155 and I157 of hBP several mutants of L3S were created: N137A, N137K, R155D, I157S and I157R. These residues differ from the corresponding residues in the sequences of hE2 and are specific for hBP only. The binding of these mutants to hE3 was determined by SPR (Table 1, Fig. 4). Substitution of I157 of hBP with serine or arginine and R155 of hBP with aspartate resulted in the significant increase in the K_D for binding with the wild-type hE3 indicating participation of these two residues in the binding to hE3. The increase in K_D for N137A and N137K of hBP was moderate (3.1 and 2.1-fold, respectively).

In a recent study the roles of several residues of hBP involved in binding with hE3 were investigated by isothermal titration calorimetry (ITC) [15]. The results of this study differ from our results, for instance, the K_D for hE3 binding to hBP determined by ITC was 0.78 nM, while the K_D determined by us using SPR was 5.26 nM. The binding of I157A of hBP was not detected by ITC while in our experiments both I157S and I157R were able to bind hE3 with 8-fold and 23-fold increase in K_D, respectively. Substitution of hBPR155 with A caused 120-fold increase in K_D measured by ITC while more drastic substitution with D in our experiments using SPR resulted in only 8.5-fold increase in K_D of binding to hE3. Binding of hBPI157A to hE3 was undetectable by ITC, however, the SPR approach used in this study revealed only a moderate decrease in the affinity of I157S and I157R for hE3. The reason for these discrepancies may lie in the experimental differences. Estimation of binding parameters by ITC is determined by thermodynamic changes during interaction of two proteins while SPR determination is based on the increase in the molecular mass when two proteins bind each other.

The results presented here show that the binding of L3S1 to hE3 is not eliminated completely by any single mutation in L3S1 tested. Although a “hot spot” theory to hE3 binding with hBP is proposed [15], our results suggest that possibly the high affinity of binding of hE3 to hBP (K_D = 5.3 nM based on SPR data) is determined not by a single very strong bond but rather by a combination of several weak electrostatic bonds and hydrophobic interactions.

4. Conclusions

The structural, fuctional and regulatory properties of the PDC have experienced evolutionary changes by incorporating additional components (such as BP, PDKs, and PDPs) to the PDCs in higher eukaryotes compared with a minimal functional bacterial PDCs. In particular, the presence of BP in higher eukaryotes has allowed monospecificity for the interactions between the central core consisting of E2 and BP and two peripheral catalytic components E1 and E3, respectively. In this report we have identified the specific residues involved in one of the major interactions between hE1 and hE2 involving hE1bD289 and hE2K276. Identification of additional electrostatic as well as hydrophobic interactions between E1 and E2 requires further investigation. In contrast, the specific binding of hE3 to hBP results from several weak electrostatic bonds and hydrophobic interactions among specific residues at the interface of dimeric hE3 with specific residues in hBP.

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Abbreviations

hPDC	human pyruvate dehydrogenase complex
E1	pyruvate dehydrogenase
E2	dihydrolipoamide acetyltransferase
E3	dihydrolipoamide dehydrogenase
BP	E3-binding protein
PDKs	pyruvate dehydrogenase kinases
PDPs	pyruvate dehydrogenase phosphatases
bs	*Bacillus stearothermophilus*
DCPIP	2,6-dichlorophenolindophenol
SPR	surface plasmon resonance
L2S	the second lipoyl domain (L2), second hinge region, E1-bindong domain (S) and third hinge region of hE2
L3S1	the lipoyl domain (L3), first hinge region, E3-binding domain (S1) and second hinge region of hBP

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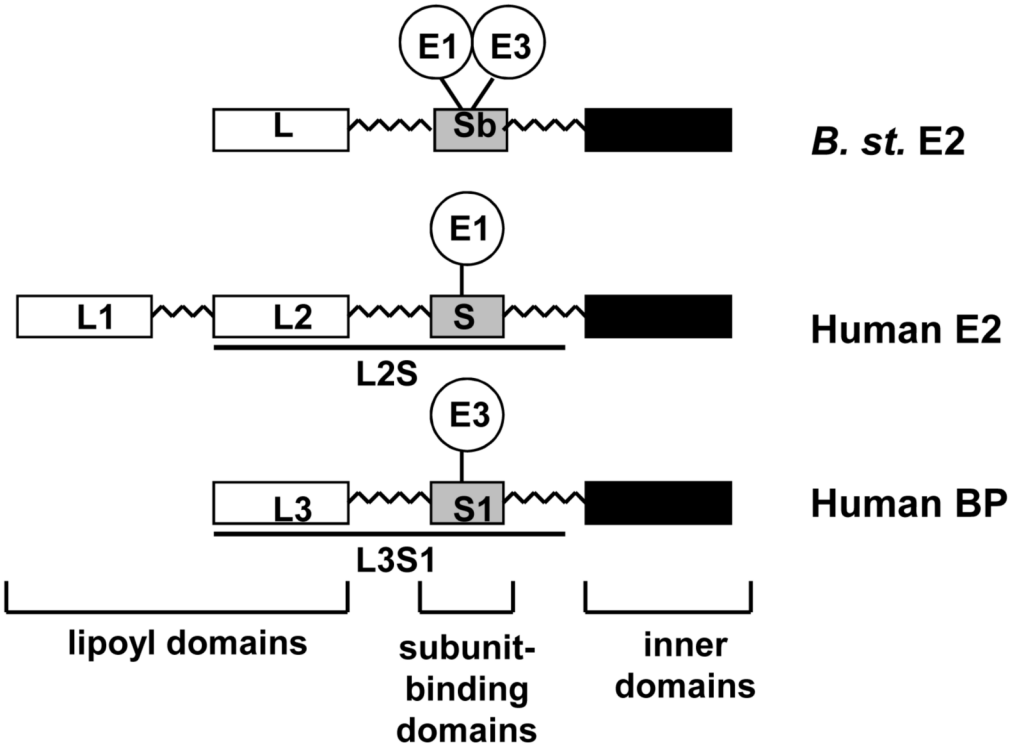

Figure 1.
Binding domains of E1 and E3 in different PDCs. L, L1, L2, L3 are lipoyl domains of bacterial E2 and human E2 and BP. Sb, S and S1 are the corresponding subunit-binding domains.

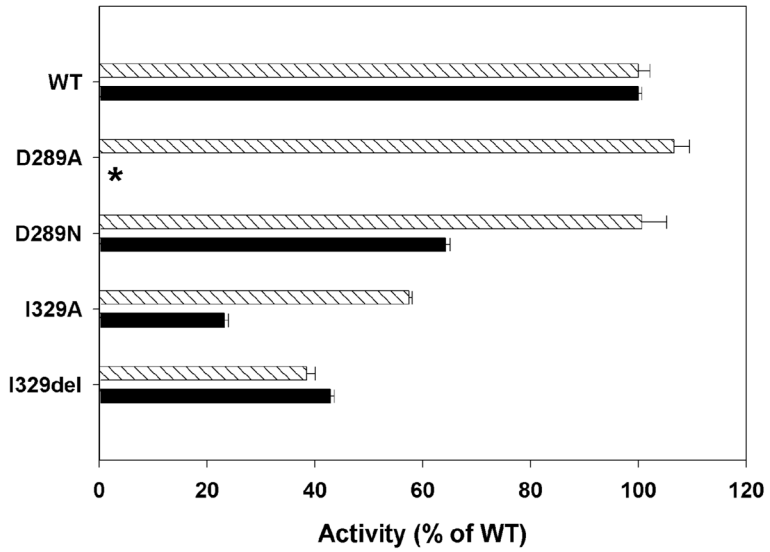

Figure 2.

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 (hatched bars) by the reduction of DCPIP. *, undetectable. Results are means ± SE (n = 4–6). Wild-type 100% activity for E1 in hPDC was 28 U/mg protein and in DCPIP-assay was 160 mU/mg protein.

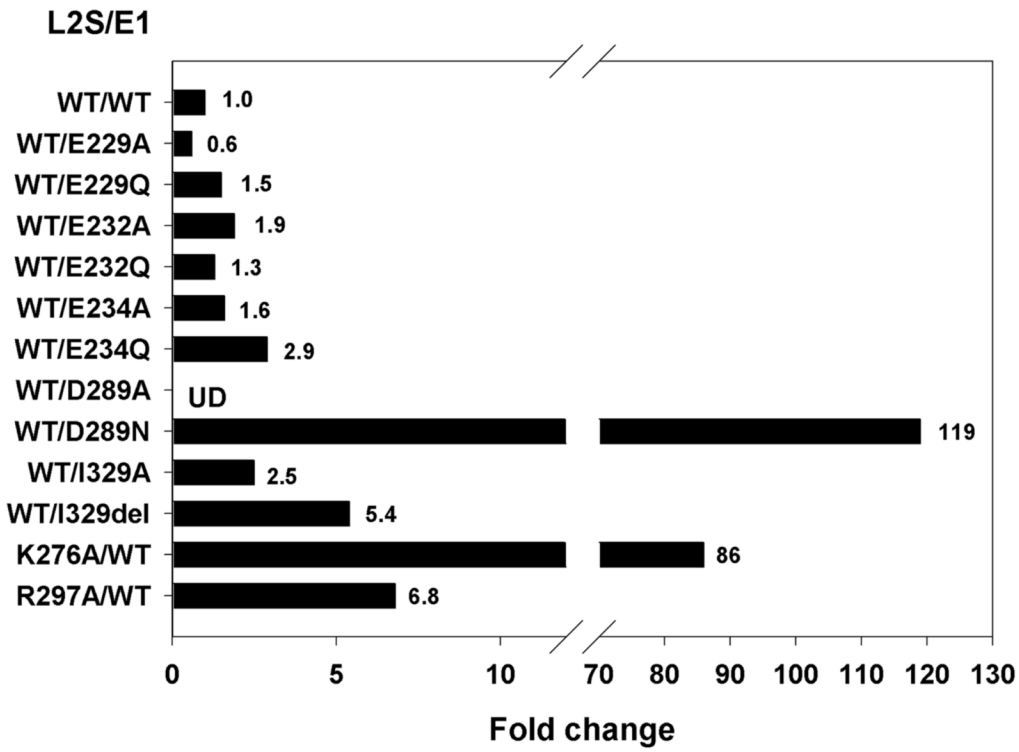

Figure 3.

Comparison of the fold increase in the K_D determined by surface plasmon resonance for the wild-type and mutant hE1s interaction with the wild-type and mutant L2Ss. Binding parameters were determined by SPR. UD, undetectable.

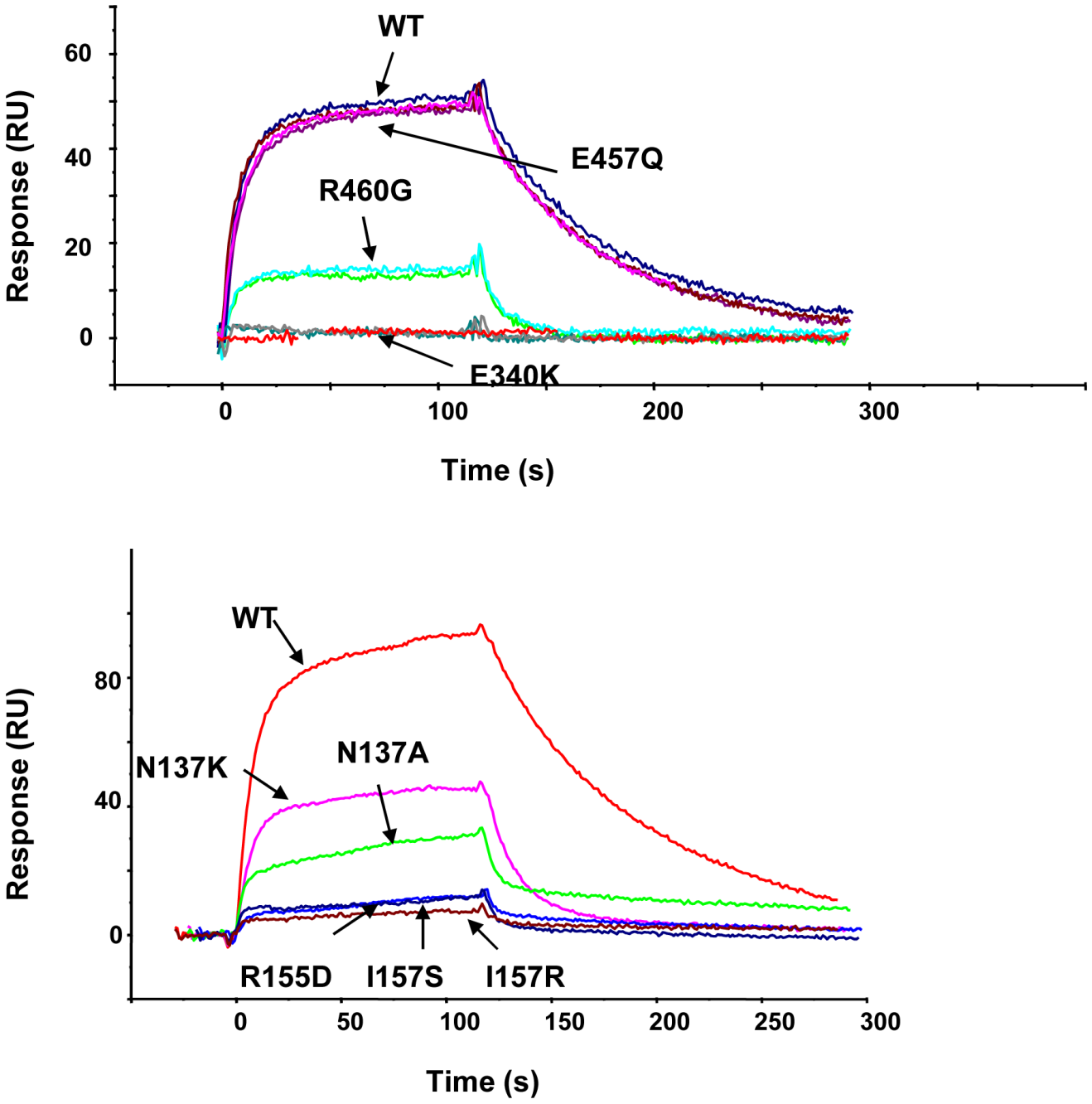

Figure 4.

Comparison of the binding of the wild-type and mutant hE3 with the wild-type L3S1 (upper panel) and binding of the wild-type hE3 with the wild-type and mutant L3S1s (lower panel). Binding of hE3 with the immobilized L3S1 was detected by SPR. E3 concentration was 50 nM (upper panel) and 39 nM (lower panel).

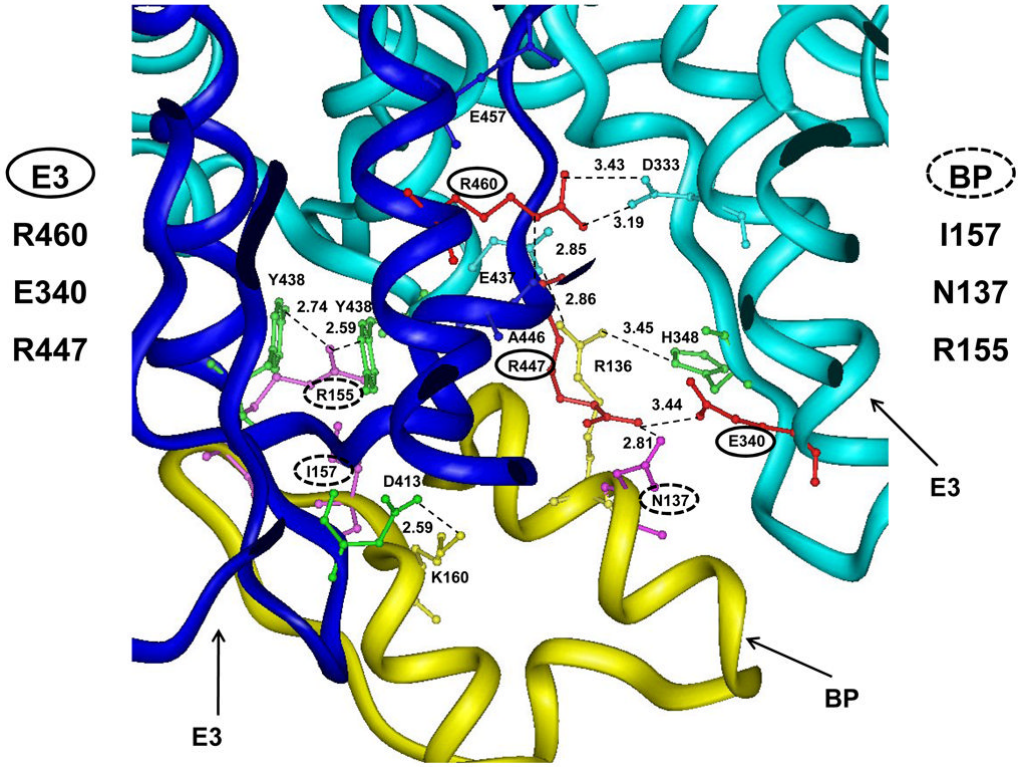

Figure 5.
Structure of hE3 with the E3-binding domain of hBP. Residues of hBP are shown in dashed circles. The pdb file 1ZY8 was used.
NOTE: Colored Figure 5 is for an on-line article and black/white Figure 5 is for a print copy.

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Table 1

Binding parameters for the wild-type and mutant hE3s interaction with the wild-type and L3S mutants of hBP.

E3	L3S	Ka 1/mMs	kd 1/s	KD nM	fold
WT	WT	2740	0.0144	5.26	1
K37E	WT	1510	0.0138	9.10	1.7
K54E	WT	1580	0.0159	10.1	1.9
P453L	WT	813	0.0173	21.2	4.0
E457Q	WT	1700	0.0145	8.54	1.6
R447M	WT	873	0.0129	14.8	2.8
K470M	WT	584	0.0151	25.9	4.9
R447M/K470M	WT	322	0.0118	36.7	7.0
R460Q	WT	134	0.065	485	92
E340K	WT	UD	UD	UD	
WT	N137A	631	0.0101	16.15	3.1
E340K	N137A	UD	UD	UD	
WT	N137K	193.5	0.0022	11.16	2.1
WT	R155D	87.75	0.0039	44.50	8.5
WT	I157S	87.80	0.0032	44.05	8.4
WT	I157R	44.8	0.0057	122.50	23
E1-WT*	WT	UD	UD	UD	

Binding parameters were determined by SPR. UD, undetectable

* The absence of binding of the wild-type E1 by the wild-type L3S is included to demonstrate specificity of binding