

Identification of the E2-binding residues in the N-terminal domain of E1 of a prokaryotic pyruvate dehydrogenase complex

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Abstract Pyruvate dehydrogenase (E1p) is one of the components of the pyruvate dehydrogenase multienzyme complex (PDHC). Previously, it was shown that the N-terminal domain of E1p is involved in its binding to the core component (E2p) of PDHC. We constructed point mutations in this domain (D17Q, D17R, E20Q, E20R, D24Q and D24R) to identify the specific residues involved in these interactions. Kinetic and binding studies show that D17 is essential for the binding of E1p to E2p. D24 is involved in the binding, but not essential, whereas E20 is not involved. None of the mutations affects the folding or dimerisation of E1p. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Pyruvate dehydrogenase multienzyme complex; Mutagenesis; Protein–protein interaction

1. Introduction

The pyruvate dehydrogenase multienzyme complex (PDHC) from Gram-negative bacteria consists of multiple copies of three different enzyme components: pyruvate dehydrogenase (E1p), dihydrolipoyl acyltransferase (E2p) and lipoyamide dehydrogenase (E3). It catalyses the oxidative decarboxylation of pyruvate, resulting in the acetylation of coenzyme A and the transfer of reducing equivalents to NAD⁺ (for reviews see [1–5]).

From N- to C-terminus, E2p consists of two or three lipoyl domains, each carrying a lipoyl-lysine group, an E1/E3 binding domain and a catalytic domain, which also forms the structural core of the complex. Flexible alanine/proline rich linkers of 20–40 amino acids interconnect these domains (for reviews on E2 see [3,5]).

The E1 component exists both as a homodimer (α_2) or a heterotetramer ($\alpha_2\beta_2$) depending on the source and type of complex. Recently the three-dimensional structure of the heterotetrameric branched-chain oxoacid dehydrogenase (E1b) from *Pseudomonas putida* and human and for homodimeric E1p from *Escherichia coli* were solved [6–8]. Moreover, recent studies have given insight into the binding mode of homodi-

meric E1p from *Azotobacter vinelandii*; limited proteolysis experiments [9] and mutagenesis studies [10] showed that the N-terminal region of E1p (amino acid 1–48) is essential for its binding to E2p. Additionally, mutagenesis experiments of E2p identified binding sites for E1p on both the E1/E3 binding domain of E2p and on the catalytic domain of E2p [11]. The construction of chimeric E2ps confirmed the importance of both these domains in the interactions between E1p and E2p [12]. For tight binding the homodimeric E1p requires two N-terminal binding regions [10], therefore it seems likely that one N-terminus of E1p interacts with the E1/E3 binding domain of E2p, whereas the other N-terminal region of E1p binds to the catalytic domain of E2p. Structure and function of the N-terminal region of E1p were studied in more detail using a synthetic peptide representing this region and it was shown that this region of E1p forms an independent folding domain that specifically competes with E1p for the binding to E2p. CD and NMR measurements indicated that the N-terminal domain is mostly α -helical [13]. These data combined with a predicted secondary structure suggest that the domain consists of a ‘helix–turn–helix’ motif, attached to the core of E1 by an extended linker or loop. The flexibility of this N-terminal domain is confirmed by the recently solved three-dimensional structure of *E. coli* E1p [8], where no interpretable electron density is observed for amino acids 1–55. Using an amino acid sequence alignment of the N-terminal region of E1p from several Gram-negative bacteria, combined with the above, we have identified the residues D17, E20 and D24 in the N-terminal domain to be likely candidates for the binding of E1p to E2p. Additionally, these residues might be involved in the species-specific binding of E1p to E2p. Here we describe a mutational analysis of these putative binding residues of E1p.

2. Materials and methods

2.1. Construction of E1p mutants

The E1p mutants D17Q, D17R, E20Q, E20R, D24Q and D24R were constructed using standard PCR mutagenesis techniques. pAFH001 [9] was used as the wild-type template plasmid DNA.

2.2. Enzyme expression and purification

The *E. coli* strain TG2, a *recA*-version of TG1 [$\Delta(lac^- proAB)$, *thi*, *supE*, *Res⁻ Mod⁻ (k)*, *F' (traD36 proA⁺B⁺, lacI^f lacZ Δ M15)] [14] harbouring the recombinant plasmid pAFH001, expressing wild-type E1p was grown and purified as described in [9]. E1p mutants were expressed and purified from *E. coli* as wild-type E1p to approximately 80% purity, omitting the gel filtration and the second ion-exchange chromatography step. The purity of the mutant E1ps was estimated from their relative intensity on SDS-PAGE [19] coloured with Co-*

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massie stain. *A. vinelandii* wild-type E2p and E3 were expressed and purified from *E. coli* TG2 as described in [15] and [16].

2.3. Enzyme activity assays

The oxidative decarboxylation of pyruvate by E1p was measured at 600 nm using 2,6-dichlorophenol-indophenol (Cl₂Ind) ($\epsilon = 21.7 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$) as an artificial electron acceptor [17]. The overall PDHC activity was reconstituted by incubating E2p with E1p or mutant E1p and E3 and was measured spectrophotometrically at 340 nm as described in [18]. Protein concentrations were estimated using the micro-biuret method [20], Bovine serum albumin was used as a standard.

2.4. Interaction of E1p and E1p mutants with E2p

The binding of E1p or mutant E1p to E2p was analysed by analytical size-exclusion chromatography using a Superose 6 column (Pharmacia Biotech) as described in [9].

3. Results and discussion

3.1. Kinetic characterisation of wild-type and mutant E1p

Based on a secondary structure prediction (Fig. 1) and on experimental data showing that the N-terminal domain has a high α -helical content, we previously proposed a ‘helix–turn–helix’ motif for this domain [13] (Fig. 2). Mainly the N-terminal α -helix has a very high content of acidic amino acid residues. The interactions between E1p and E2p are highly species-specific, and it is therefore not likely that conserved residues are solely involved in these interactions. Residues D17, E20 and D24 of *A. vinelandii* E1p are not conserved and moreover, the construction of a helical wheel of amino acid residues 9–25 shows that they are all on the same side of the helix. It therefore seems very likely that (some of) these residues are involved in the interactions between E1p and E2p. To test this hypothesis, we constructed the following mutants of E1p: E1p-D17Q, E1p-D17R, E1p-E20Q, E1p-E20R, E1p-D24Q and E1p-D24R.

Table 1 shows the kinetic parameters of these mutants and of wild-type E1p. Clearly, the mutations D17R, E20Q, E20R and D24Q have no significant effects on the kinetic properties of the enzyme. The mutations D17Q and D24R on the other hand, do change the kinetics of the enzyme. Especially E1p-D24R is strongly impaired in its E1-activity, whereas the effect on E1-D17Q is intermediate. Removal of amino acids 1–48 affects the affinity of E1p for pyruvate [10] and it is therefore not entirely surprising that mutations in this region affect the kinetic parameters of E1p. From the structural and functional information available at this time it is not possible to give an explanation of this effect. Results shown below prove however that global misfolding of these mutants does not cause the effect on the activity of E1p-D24R and E1p-D17Q and therefore it is likely that the effects on the catalytic activity of these mutants is not directly correlated with their ability to interact with E2p.

3.2. Binding properties of wild-type and mutant E1p

Two different methods were used to study the binding of wild-type and mutant E1p to E2p. Firstly, PDHC was reconstituted by the addition of wild-type or mutant E1p to the E2p/E3 sub-complex and PDHC activity was measured (Table 1, Fig. 3). Secondly, the binding properties of wild-type and mutant E1p were studied by analytical gel filtration (Table 1).

3.3. Binding studies by PDHC reconstitution

The first method shows that at an E1p:E2p ratio of 5:1 wild-type E1p virtually saturates E2p (based on the PDHC-activity). E1p-E20Q and E1p-E20R also saturate E2p at this ratio and consequently the residue E20 does not seem to be involved in the interactions between E1p and E2p. Addition of E1p-D17R, E1p-D17Q or E1p-D24R on the other hand, results in only 1%, 28% or 10% of maximum PDHC activity at a 5:1 E1p:E2p ratio (Table 1). The mutation D24Q has an intermediate effect on the PDHC activity at this ratio. At a 60-fold excess of E1p the PDHC activity of the severely affected E1p mutants (E1p-D17R and E1p-D24R) reaches about 50% of maximum PDHC activity. E1p-D17Q almost saturates E2p at this ratio, whereas the mutant E1p-D24Q attains saturation of E2p at 16-fold excess. Since at a 60-fold excess of E1p the PDHC-activity curves of the severely affected E1p mutants do not reach saturation, but are still increasing, it seems likely that at infinite mutant E1p concentrations saturation may occur.

Comparing the effects of the mutations D17Q and D17R, one sees that even though E1p-D17Q is quite impaired in its E1 activity the mutation D17R (which has no effect on the E1-activity) has a stronger effect on the complex activity. Consequently, the impaired E1 activity of E1p-D17Q cannot (fully) cause the decrease in complex activity. Similar considerations are valid for E1p-D24R and E1p-D24Q; here it is also likely that the effect of E1p-D24Q on the complex activity is (at least partly) caused by effects on the binding properties of this mutant.

3.4. Binding studies by analytical gel filtration

Free *A. vinelandii* E2p forms a 24-meric cubic core structure of about 1.5 MDa. Upon binding of wild-type E1p or E3 the core dissociates into trimers with E1p or E3 bound (~ 400 – 800 kDa). These two forms of E2p can be separated by gel filtration chromatography [9]. The results obtained by this method (Table 1) correlate very well with the results that we described above. The absence of dissociation of E2p by E1p-D17Q, E1p-D17R or E1p-D24R is analogous to that observed in Fig. 2 at ‘low’ (2:1) E1p:E2p ratio; no, or very low PDHC activity is measured at this ratio using these mutants. These experiments confirm that the low PDHC activities of E2p/E3 sub-complex recombined with either E1p-D17Q or E1p-D24R

<i>A. vin</i>	MQDMDLDPPI	ETQEWLDSLE	SVLDEEGEER	AHYLLTRMGE	LATRTGTQLP
<i>P. aer</i>	MQDLDPV	ETQEWLDALE	SVLDRREGDR	AHYLMTRMGE	LASRSQTQLP
<i>A. eut</i>	MSAVPEQILGASSANDAPQ	ETHEWLDAEQ	GVLAEEGPAR	AAFLIDKQIE	YARNNGVTQP
<i>N. men</i>	MSTQLHDVDPI	ETQEWLDALS	SVLEYEGGER	AQYLLLENLVK	YCRDKGVRMP
<i>E. coli</i>	MSERFPNDVDPI	ETRDWLQAIE	SVIREEGVER	AQYLLDQLLA	EARKGGVNVA
<i>H. inf</i>	MSEILKNDVDPI	ETQDWLQSLD	SLIREEGVER	AQYIVEQVIG	QARTSGVSLP

Fig. 1. Sequence alignment of the N-terminal sequence of E1p from several Gram-negative bacteria. Entirely conserved negatively charged amino acid residues are boxed and the mutated residues are shaded in grey. *A. vin*, *A. vinelandii* E1p; *P. aer*, *P. aeruginosa* E1p; *A. eut*, *Alcaligenes eutrophus* E1p; *N. men*, *Neisseria meningitidis* E1p; *E. coli*, *E. coli* E1p; *H. inf*, *Haemophilus influenzae* E1p.

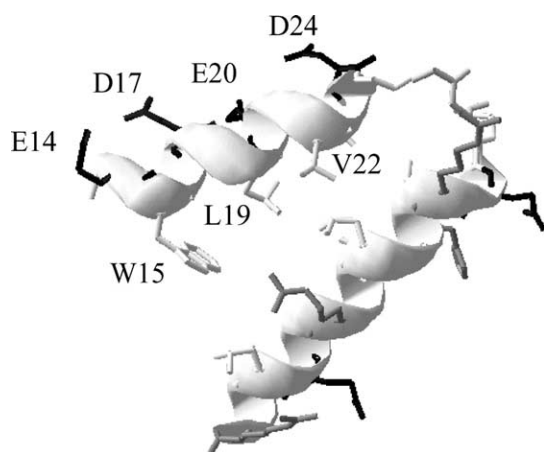


Fig. 2. A model of the N-terminal domain of E1p as inferred from a secondary structure prediction and NMR, CD and fluorescence spectroscopy data [13].

are not caused by their decreased E1 activity, but (at least partially) by a decreased binding affinity for E2p. Even though binding of E1p-D24Q to E2p results in dissociation of E2p, a considerable fraction of E1p-D24Q remains unbound (data not shown), which correlates well with the intermediate effect that this mutant has on the complex activity as was measured above.

The elution volumes of all six mutant E1ps are identical to the elution volumes of wild-type E1p. This demonstrates that none of the mutations affects the folding or dimerisation of E1p (as the molecular sizes of the mutants are not affected). This important observation shows that the observed effects on the activity and on the binding properties of these mutants are not caused by misfolding of E1p. Dimerisation of E1p is crucial for the binding of E1p to E2p and these results rule out that effects on the binding are caused by an indirect effect via an impaired dimerisation. Summarising, the above-described measurements clearly establish that D17 is essential for the binding of E1p to E2p. D24 on the other hand seems to be involved in the interactions, but does not seem to be essential. Mutation of the middle amino acid residue (E20) of the acidic 'patch' has no influence on the interactions of E1p to E2p. This last observation indicates that the effects on D17 and D24 are specific and not due to local misfolding.

3.5. Model of the binding mode of E1p to E2p

Using the above-described results we have refined the model

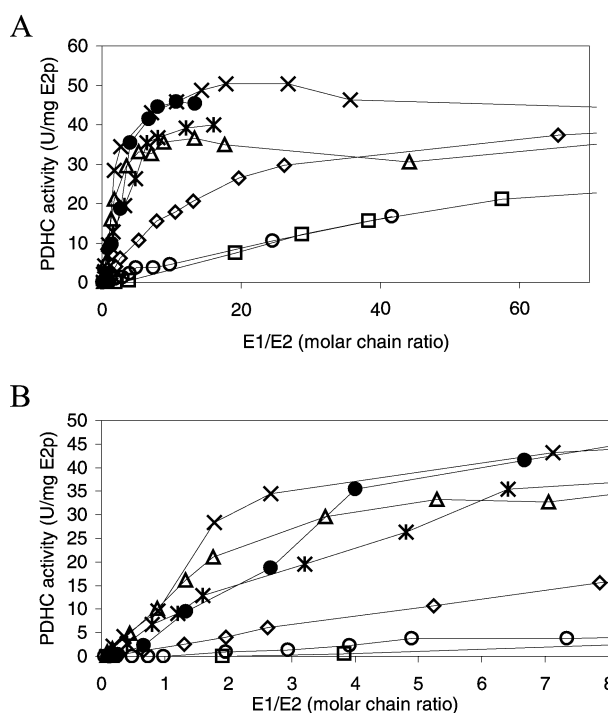


Fig. 3. A: Reconstitution of *A. vinelandii* PDHC using wild-type E2p (3.9 μ M) and E3 (2.8 μ M) and either wild-type or mutant E1p. E2p is pre-incubated with E3 at a molar ratio of 1:0.7 (E2p:E3). Subsequently varying amounts of E1p are added to restore complex activity. E1p-D17Q (\diamond), E1p-D17R (\square), E1p-E20Q (\triangle), E1p-E20R (\times), E1p-D24Q ($*$), E1p-D24R (\circ) and wild-type E1p (\bullet). B: Idem at low E1/E2 chain ratios.

of the binding mode of E1p and E2p that was postulated before [13] (Fig. 2). A helical wheel of helix I of the proposed 'helix–turn–helix' motif (amino acids 9–25) shows that the residues D17, E20 and D24 are on one side of the helix, whereas on the opposite side W15, L19 and V22 form a hydrophobic 'patch'. Helix II, of which it is less evident where it starts and finishes, also has a hydrophobic side, which can form a hydrophobic 'pocket' with the residues of helix I. As shown above, we have established that E20 is not involved in interactions between E1p and E2p. D24 likely is (indirectly) involved in the interactions between E1p and E2p, whereas D17 is an essential binding residue. None of the mutations result in complete abolishment of the binding capacity of E1p to E2p; neither do point mutations in E2p [11]. Apparently

Table 1

Summary of the kinetic parameters for the E1-reaction by *A. vinelandii* E1p and *A. vinelandii* E1p mutants and the binding properties of wild-type and mutant E1p as determined by reconstitution of PDHC activity and analytical gel filtration

	$S_{0.5}$ (μ M)	V_{max} (mU mg ⁻¹)	Hill coefficient	E1p:E2p ratio 5:1 [U mg E2p ⁻¹ (% of control)]	E1p:E2p ratio 60:1 [U mg E2p ⁻¹ (% of control)]	Dissociation of E2p
E1p (wt)	24 \pm 7	55 \pm 3	1.1 \pm 0.1	40 (100)	45 (112) ^a	yes
D17Q	24 \pm 8	20 \pm 2	0.5 \pm 0.2	11 (28)	38 (95)	no
D17R	21 \pm 4	38 \pm 1	1.0 \pm 0.1	0.5 (1)	21 (53)	no
E20Q	8 \pm 0.9	58 \pm 1	1.2 \pm 0.1	33 (83)	32 (80)	yes
E20R	20 \pm 3	90 \pm 3	0.9 \pm 0.1	43 (107)	43 (107)	yes
D24Q	21 \pm 5	63 \pm 3	1.0 \pm 0.1	26 (65)	40 (100) ^a	yes
D24R	14 \pm 3	11 \pm 0.4	ND	4 (10)	17 (43)	no

$S_{0.5}$ and V_{max} values are calculated from V vs. $[S]$ curves. The Hill coefficients are calculated from $\log([S])$ vs. $\log((V_{max}-v)/v)$ curves. The activities of pyruvate dehydrogenase complexes containing wild-type E2p and E3 from *A. vinelandii* and wild-type or mutant E1p are shown at an E1p:E2p ratio of 5:1 and 60:1. The reactions were performed as described in the experimental procedures. ND, not determined.

^aDetermined at a E1p:E2p ratio of 16:1.

the binding interface is composed of more than one region both on E2p (binding domain and catalytic domain) and on E1p.

3.6. Species specificity

Not only from their proposed location in the first helix of the N-terminal domain, but also based on species differences, it seems likely that D17, E20 or D24 is involved in the binding of E1p to E2p. Previously, it was shown that the binding of E1p to E2p is highly species-specific [12]. Based on this, it seems likely that the binding residues in the N-terminal binding domain of E1p are not entirely conserved, i.e. difference between species should be observed. Whereas the surrounding acidic residues (E11, E14 and E26) are highly conserved, D17, E20 and D24 of *A. vinelandii* E1p exhibit differences with some of the other species (Fig. 1). In *E. coli* E1p these residues are Q17, E20 and R24. Previously, it was shown, using chimeras of *E. coli* and *A. vinelandii* E2p that *E. coli* E1p solely binds to *E. coli* E2p and not at all to *A. vinelandii* E2p. Binding, on the other hand, of, *vis a versa*, *A. vinelandii* E1p to *E. coli* E2p still results in 60% residual PDHC activity. Testing the reverse effect (whether the mutations D17Q and D24R result in an increased binding to *E. coli* E2p) is extremely difficult, as it would require *E. coli* E2p that contains absolutely no *E. coli* E1p. The fact that the mutations D17Q and D24R both have such a strong effect on the binding abilities of E1p nevertheless correlates well with the fact that *E. coli* E1p binds very poorly to *A. vinelandii* E2p.

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