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Time-resolved fluorescence studies on mutants of the dihydrolipoyl transacetylase (E2) component of the pyruvate dehydrogenase complex from *Azotobacter vinelandii*

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Fluorescence anisotropy decays were measured for the wild-type dihydrolipoyl transacetylase (E2) component of pyruvate dehydrogenase complex from *Azotobacter vinelandii* and *E. coli* and for E2-mutants from *A. vinelandii* in which the alanine-proline-rich sequence between the binding domain and the catalytic domain is partially or completely deleted. In both E2-mutants the rotational mobility of the lipoyl domain and the overall activity after reconstitution of the complex are significantly decreased indicating the important role of the deleted sequence for the movement of the lipoyl domain and the transfer of substrates between the different active sites within the complex.

Dihydrolipoyl transacetylase; Pyruvate dehydrogenase complex; Time-resolved fluorescence; Mobility

1. INTRODUCTION

The dihydrolipoyl transacetylase (E2) is the core component of the pyruvate dehydrogenase complex (PDC) which catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA. The isolated E2-component from PDC of *Azotobacter vinelandii* and *E. coli* consists of 24 subunits. Upon binding of the peripheral components pyruvate dehydrogenase (E1) and lipoamide dehydrogenase (E3), the E2 from *A. vinelandii*, behaves in a unique way as it dissociates into tetramers [1,2].

The domain structure of the E2 from *A. vinelandii* is shown in Fig. 1. The N-terminal part, called lipoyl domain, consists of three homologeous repeating sequences [3] of about 80 amino acid residues, each of which contains a lysyl residue as a potential covalent attachment site for a lipoyl moiety. These lipoyl subdomains form compact structures which can be isolated individually by proteolytic digestion [4]. After limited proteolysis with trypsin the lipoyl domain can be separated from the catalytic domain, which contains the acetyltransferase active site and the polymer interface. A less compact region with protease-sensitive sites is located between the two larger domains. In this region the binding sites for E1 [5] and E3 [6] are located. The E2 domains and the repeating subdomains within the lipoyl domain are separated from each other by sequences of 20–30 residues, that are very rich in alanine, proline and charged amino acids (apa-sequences, Fig. 1).

In the intact complex the acetyl groups and reducing equivalents are transferred between the different active sites by lipoyllysyl residues, which act as swinging arms [7]. It was suggested, that the lipoyllysyl residues alone are too short to reach all catalytic centres [8]. Therefore a part of the protein chain around the lipoyl residues is thought to be flexible. For the apa-sequences a high conformational flexibility could be demonstrated by ^{1}H-NMR [9,10]. By fluorescence anisotropy of IAANS-labelled lipoyl groups it was shown that, although all 4 apa-regions may possess internal flexibility, the three repeating sequences of the lipoyl domain move together, possibly around a hinge in the apa-sequences flanking the binding domain (apa-3 or -4). This movement is restricted by the binding of the peripheral components [11].

In this paper, fluorescence anisotropy decay experiments are described with mutants of E2 from *A. vinelandii* in which the apa-sequence between the binding domain and the catalytic domain (apa-4) is partially or completely deleted. In comparison to the wild-type enzymes from *A. vinelandii* and *E. coli* the mobility of the lipoyl domains is significantly decreased in the E2-mutants.

2. MATERIALS AND METHODS

Restriction endonucleases, DNA polymerase I (Klenow fragment), T4-DNA-ligase, S1-nuclease, calf intestinal alkaline phosphatase and

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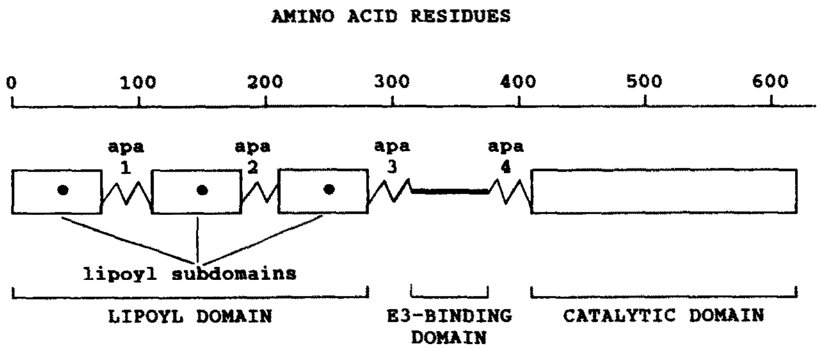
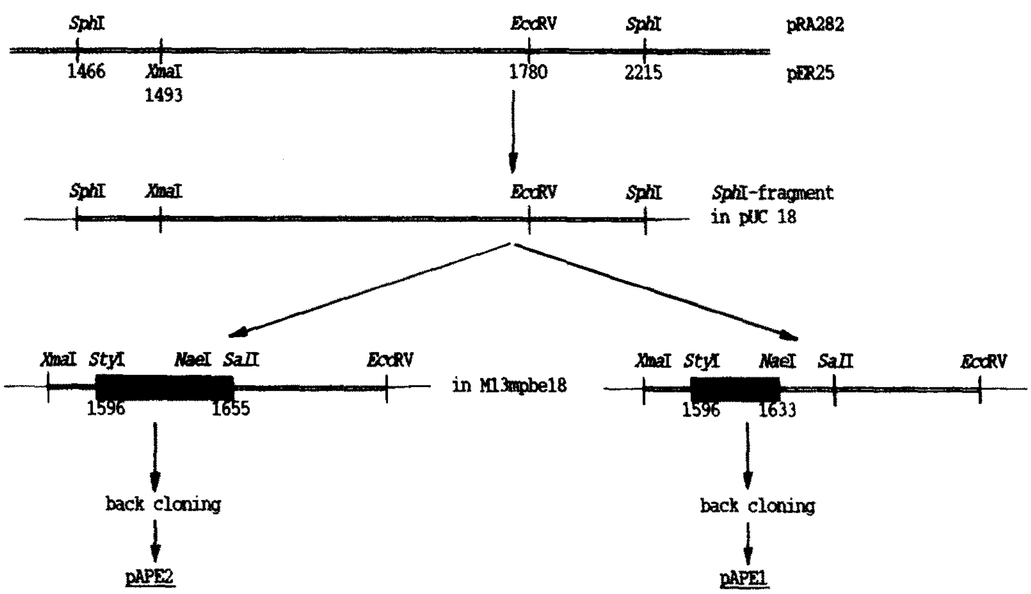


Fig. 1. Domain structure of the dihydrolipoyl transacetylase from *A. vinelandii*. The lipoylation site within the lipoyl domains is indicated by a filled circle (●). Tilted lines represent parts of the E2 chain rich in alanine, proline and charged amino acids (apa-1–4).

A



B

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                    E1/E3-
                binding domain¦         Ala-Pro-region          ¦       catalytic domain
A.vin. wt     V K A M M Q K A K E A P A A G A A S G A G I P P I P P V D F A K Y G E I E E V P M T R L  417
A.vin. pAPE1  V K A M M Q K A K - - - - - - - - - - - - G I P P I P P V D F A K Y G E I E E V P M T R L  406
A.vin. pAPE2  V K A M M Q K A - - - - - - - - - - - - - - - - - - - - - F A K Y G E I E E V P M T R L  396
              * *       *   * * * *         *     *   *   * * *   *   * * * * * *       *
E.coli wt     V K E A I K R A E A A P A A T G G G I P G M L P W P K V D F S K F G E I E E V E L G R I  407
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Fig. 2. (A) Subcloning procedure for the generation of deletions in the alanine-proline-rich region N-terminal of the catalytic domain of E2 from *A. vinelandii*. The double line indicates the fragment from pRA282 inserted into the subcloning vectors. The deleted parts of the sequence are given by filled boxes. The numbers for the restriction sites correspond to the base pairs in the original E2-sequence [5]. (B) Amino acid sequences of the alanine-proline-rich part (apa-4) and of the N-terminus of the catalytic domain of wild-type E2 from *A. vinelandii* [5] and *E. coli* [18] and of the mutated E2-proteins. The sequence number of the last residue in each line is indicated to the right. Homologeous residues between the wild-type enzymes of *A. vinelandii* and *E. coli* are given by asterisks. Deleted amino acids are indicated by a bar.

universal M13 sequencing primer were purchased from Bethesda Research Laboratories (BRL) or Boehringer. [α-^{32}P]dATP (3000 Ci/mmol) was obtained from New England Nuclear and 2-(4-iodoacetamidoanilino)-naphthalene-6-sulfonic acid (IAANS) was purchased from Molecular Probes.

E. coli strain TG2, a *recA*$^-$ version of TG1 [Δ(*lac-pro*), *thi*, *supE*, [Res$^-$ Mod$^-$ (k)], F' (*traD36 proA*$^+$*B*$^+$, *lacI*^{q} *lacZ* ΔM15)] was used throughout [12]. Plasmid pUC 18 [13] and phages M13mp18 [14] and M13mpbe18 [15] were used for subcloning and sequencing. Standard DNA operations were performed as described in [16].

The subcloning strategy and the amino acid sequences of the alanine-proline-rich region N-terminal of the catalytic domain for all E2-proteins studied in this paper are given in Fig. 2. For the construction of the E2-mutants the plasmid pRA282 [3], containing the E2-gene from *A. vinelandii* was used. By site-directed mutagenesis a *SmaI*/*XmaI*-site was generated at position 1493 of the original sequence without influencing the properties of the E2. The resulting plasmid was named pER25. From pER25 a 750 bp *SphI*-fragment was cut out and ligated into the *SphI*-site of pUC18 in which the *XmaI*-site in the polylinker was destroyed [17]. From this plasmid a 290 bp *XmaI*/*Eco*RV fragment was isolated and ligated in the corresponding sites of M13mpbe18. The mutation for pAPE2 was performed by digesting the M13mpbe18 RF-DNA, containing the *XmaI*/*Eco*RV insert, with *StyI* and *SalI*. Both restriction sites were made blunt-ended by S1-nuclease and ligated. For pAPE1 the M13mpbe18-DNA with the *XmaI*/*Eco*RV insert was digested by *StyI*, the restriction site was filled using the Klenow fragment of DNA-polymerase I and next the DNA was digested by *NaeI*. After ligation the deletions were checked by sequencing using the dideoxy-chain-termination method of Sanger [18]. For both mutations the described subcloning procedure was performed backwards and finally the ligation of the *SphI*-fragments containing the deletions shown in Fig. 2B into pRA282 was checked by E2-activity measurements and SDS-gel electrophoresis of cell-free extracts after the expression of the proteins. In pAPE1 the amino acid residues 383–393, in pAPE2 the residues 382–402 are deleted.

The gene encoding E2 of the pyruvate dehydrogenase complex from *E. coli* has been cloned and sequenced [19]. We recloned the gene into pUC9 and the resulting plasmid was named pAW10 [17]. *A. vinelandii* wild-type E2 and the mutated proteins as well as *E. coli* E2 were purified from *E. coli* TG2 (pRA282), TG2 (pAPE1), TG2 (pAPE2) and TG2 (pAW10) by the method described in [20]. All E2 samples were finally purified by gel filtration (FPLC) on Superose-6 in 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride. The lipoamide dehydrogenase (E3) was purified from *E. coli* TG2 (pAW104) according to [21].

Labelling of the E2 samples (5 mg of each enzyme) was carried out as described in [11]. For complexation the labelled E2 samples were incubated for 45 min with E3 in a molar ratio of 2:1 (E3/E2). At this E3 concentration both the E1 and the E3-binding sites on E2 are saturated by E3.

Time-resolved fluorescence decay was measured using a system consisting of a frequency-doubled synchronously pumped dye laser for excitation and time-correlated single photon counting for detection as described [11,22]. The excitation wavelength was 340 nm and the emission was monitored via a 450 nm band-pass filter (Balzers K 45). All experiments were carried out at 20°C. Data analysis was performed as described in [23].

3. RESULTS AND DISCUSSION

The pAPE1 and pAPE2-encoded E2-mutants were purified as described for the wild-type enzyme [20]. For both mutants no differences in the specific E2-activity and in the intersubunit interaction (24meric structure) could be detected (results not shown).

The fitted fluorescence anisotropy decay curves of wild-type E2 from *A. vinelandii* and *E. coli* and of the E2-mutants encoded by pAPE1 and pAPE2 are shown in Fig. 3A. The exponential decays derived from the fits are listed in Table I. For wild-type E2 from *A. vinelandii* the anisotropy decay curve can be described as a triple exponential function with a short (0.46 ns), a longer (14.7 ns) and a very long (600 ns) time constant. The correlation times for the short component (free rotation of the lipoyl group around the linkage with the protein chain) and the longer time constant (rotation of the whole lipoyl domain) correlate well with the values found in previous fluorescence anisotropy experiments of E2. The latter value seems to exclude involvement of the apa-regions between the lipoyl subdomains [11]. The very long correlation time representing the rotation of the whole protein (1.6 MDa) is fixed in the fitting procedure and can be calculated on the basis of an empirical formula relating the correlation time ϕ with the molecular weight (M_r) of a hydrated, spherical polypeptide at 20°C: $\phi(\text{ns}) = 3.84 \times 10^{-4} \times M_r$ [24]. From this formula the correlation time ϕ_3 for all E2 samples was

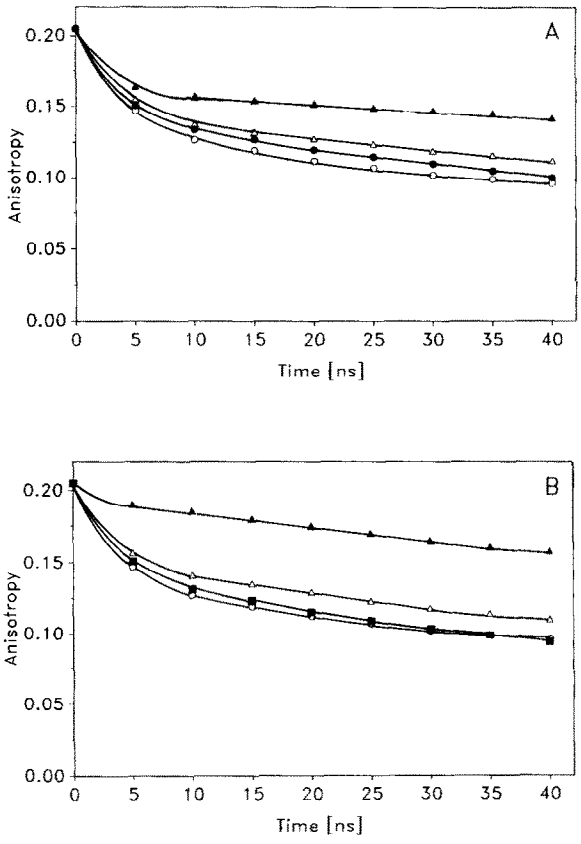

Fig. 3. Fluorescence anisotropy decay (fitted curves) of wild-type and mutated E2 from *A. vinelandii* and of wild-type E2 from *E. coli*. (A) *A. vinelandii* wild-type E2 (○); *E. coli* wild-type E2 (●); pAPE1 (▲); pAPE2 (△). (B) The E2 samples from *A. vinelandii* were incubated for 45 min with E3 in a molar concentration (E2/E3) of 4:8 (both the E3- and the E1-binding sites are saturated by E3). *A. vinelandii* wild-type E2 (○); *A. vinelandii* wild type E2 + E3 (■); pAPE1 + E3 (▲); pAPE2 + E3 (△). The parameters of the triple exponential decays and the standard errors derived from the fits are listed in Table I.

Table I

Fluorescence decay parameters of wild-type and mutant E2 from *A. vinelandii* and of wild-type E2 from *E. coli* with and without complexation with E3

Exp.	Protein[a]	M_r (kDa)	β_1	ϕ_1 (ns)	β_2	ϕ_2 (ns)	β_3	ϕ_3[b] (ns)	S_1[c]	θ_c[d]	S_2[e]
1	A.v.E2 wt	1560	0.06±0.003	0.46±0.110	0.05±0.002	14.7±1.30	0.07±0.001	600	0.82±0.10	38±5	0.66±0.08
2	E.c.E2 wt	1580	0.05±0.002	1.40±0.130	0.02±0.005	24.8±4.80	0.14±0.004	600	0.87±0.12	32±7	0.93±0.15
3	A.v.E2 pAPE1	1535	0.03±0.001	0.50±0.008	0.06±0.008	48.2±5.60	0.07±0.004	590	0.90±0.13	27±9	0.73±0.09
4	A.v.E2 pAPE2	1500	0.05±0.002	1.02±0.100	0.07±0.010	31.5±4.82	0.08±0.005	570	0.86±0.09	35±6	0.73±0.07
5	A.v.E2 wt + E3	660	0.04±0.004	0.53±0.008	0.07±0.004	18.4±2.35	0.08±0.003	253	0.88±0.11	32±9	0.73±0.08
6	A.v.E2 pAPE1 + E3	656	0.02±0.001	0.32±0.004	0.06±0.008	56.3±7.89	0.09±0.01	252	0.93±0.14	25±10	0.77±0.12
7	A.v.E2 pAPE2 + E3	650	0.05±0.002	1.06±0.100	0.08±0.010	33.6±4.81	0.08±0.01	251	0.87±0.12	33±8	0.70±0.09

[a]See section 2 and legend to Fig. 3 for details. [b]Fixed in the analysis. [c]From $(S_1)^2 = \frac{\beta_2 + \beta_3}{\beta_1 + \beta_2 + \beta_3}$. [d]From $(S_1)^2 = 1/2\cos\theta_c\,(1 + \cos\theta_c)$. [e]From $(S_1)^2 = \frac{\beta_3}{\beta_2 + \beta_3}$

calculated. The short component ϕ_1 (free rotation of the lipoyl group) was found for all E2 samples in the same range (around 1 ns).

The time constant for the rotation of the whole lipoyl domain (ϕ_2) increased in the E2-mutant (pAPE1) with the deletion of the N-terminal half of the apa-4-sequence to 48 ns. The increase of the order parameter S_1 and the strong decrease of the related cone angle Θ_c that can be derived from a wobbling-in-cone model [25] indicates that the motion of the label becomes restricted in this mutant. After the complete deletion of apa-4 (pAPE2) the correlation time ϕ_2 was found ranging between the values for the wild-type E2 and the protein encoded by pAPE1. The following explananation is possible for this effect. In the wild-type E2 from *A. vinelandii* a hydrophobic interaction could exist between both apa-3 and apa-4 which can explain the relatively high mobility of the lipoyl domain (15 ns) by a common and stimulated action of both apa-sequences. Deletion of 50% of apa-4 in the pAPE1-encoded protein could result in a change of the interaction between the two apa-sequences (lower hydrophobicity of apa-4; the N-terminus of the catalytic domain or the binding domain can be located more closely to the mobile region). This could lead to a restriction of the mobility of apa-3, resulting in an increase of the correlation time to 48 ns. Binding of E3 to the pAPE1-encoded E2-mutant resulted in a further increase of the correlation time ϕ_2 and the order parameter S_1 as well as in a slight decrease of the cone angle. This could be explained in terms of a further restriction of the mobility, caused by a structural change of the binding domain after binding of E3 or by E3 itself. In the pAPE2-encoded protein, where the whole apa-4 sequence is deleted, the ϕ_2 of 31.5 ns represents the mobility in apa-3, which is only weakly changed after binding of E3.

The E2 from *E. coli* shows especially in the second part of the apa-4 sequence a lower homology with the E2 from *A. vinelandii* (Fig.2B). The length of the apa-4 sequence between the E1/E3-binding domain and the catalytic domain is not different in *E. coli* E2, but the hydrophobicity is lower and comparable with that of the pAPE1-encoded E2-mutant. Because of the intact spacer function of apa-4 in the *E. coli* E2 the supposed interaction between apa-3 and apa-4 might be weaker and the correlation time ϕ_2 assumes a value between wild-type E2 from *A. vinelandii* and the pAPE1-encoded mutant.

Reconstitutions of PDC using the E2-mutants encoded by pAPE1 and pAPE2 as core enzymes showed, that the overall activity of the complex is decreased to 80% (45 U/mg E2) after deletion of the first half of the apa-sequence (pAPE1), whereas for pAPE2 (complete deletion of the apa-sequence) only about 50% (27 U/mg E2) of the wild-type activity (56 U/mg E2) in the reconstituted complex could be detected. That means, that there is no direct correlation between the decrease of the mobility of the lipoyl domain and the complex activity.

Deletion of 13 out of 32 residues of apa-3 from an *E. coli* E2-mutant with only one lipoyl subdomain did not effect specific catalytic activity of the complex [26]. Further deletion up to 31 of the 32 residues resulted in a fall of the specific catalytic activity of the PDC to around 20% and in a substantial decrease of the reductive acetylation of the lipoyl domains and corresponding losses of active site coupling [27].

From the fluorescence anisotropy experiments on the pAPE1 and pAPE2-encoded proteins and the results from the deletions in apa-3 of *E. coli* it can be predicted, that both apa-3 and apa-4 are important for the mobility of the lipoyl domains and the transfer of substrates between the active sites in the complex. Complete deletion of either one of these apa-sequences results in a significantly decreased complex activity, but the other apa-region flanking the binding domain can retain a value of mobility which is sufficient for the function of the swinging lipoyl arms in the complex.

Further studies of the properties of the isolated E2-mutants encoded by pAPE1 and pAPE2 and of these enzymes in reconstituted complexes as well as

deletions in the apa-sequence between lipoyl domain and binding domain (apa-3) will be performed to describe more detailed the role of the mobility of the lipoyl domains and the specificity of the hinge regions for this movement and for the function of the complex.

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