Time-resolved fluorescence studies on the dihydrolipoyl transacetylase (E₂) component of the pyruvate dehydrogenase complex from \textit{Azotobacter vinelandii}

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The dihydrolipoyl transacetylase (E₂) component of \textit{A. vinelandii} PDC and its lipoyl domain shows similar dynamic properties as revealed with fluorescence anisotropy decay of lipoyl-bound IAANS. The lipoyl domain (32.6 kDa), containing three almost identical subdomains shows a mode of rotation characteristic for a protein of about 30 kDa. A similar rotation is found in E₂, indicating an independent rotational mobility of the whole domain in the multimeric E₂ core (1.6 MDa). No independent rotation of a single lipoyl subdomain (10 kDa) is observed. The E₂ component, in contrast to the E₃ component, shows interaction with the lipoyl domain.

Dihydrolipoyl transacetylase; Pyruvate dehydrogenase complex; Time-resolved fluorescence; Mobility; (\textit{Azotobacter vinelandii})

1. INTRODUCTION

The pyruvate dehydrogenase complex catalyzes the oxidative decarboxylation of pyruvate to acetyl CoA. The structural core of the \textit{A. vinelandii} complex is composed of four dihydrolipoyl transacetylase (E₂) chains, to which three dimers of pyruvate dehydrogenase (E₁) and one dimer of lipoamide dehydrogenase (E₃) are bound [1]. After removal of these peripheral components the E₂ core aggregates to a cubic 24-meric structure [2,3]. The substrates are transferred between the different components by lipoyllysine residues, which act as swinging arms between the different active sites [4]. In \textit{E. coli} and in \textit{A. vinelandii} E₂ the N-terminal part of the E₂ chain consists of three homologous repeating sequences [5,6]. Each repeat of about 80 amino acid residues contains a lysyl residue that is a potential site for lipoylation. Each repeat is separated from its neighbour by a region of about 20 residues, very rich in alanyl and prolyl residues. After limited proteolysis of E₂ with trypsin this N-terminal part, called lipoyl domain (32.6 kDa), is separated from a part called the catalytic domain, which forms the multimeric core [7]. In \textit{E. coli} it is shown that after limited proteolysis with \textit{Staphylococcus aureus} V8 proteinase the three repeats can be isolated separately as folded subdomains [8]. In other organisms such as Gram-positive bacteria and eukaryotes, and also in the closely related 2-oxoglutarate dehydrogenase complex, only one lipoyl subdomain is found [9–11]. Spin label experiments with the PDC from \textit{E. coli} have demonstrated that the dithiolane ring of the lipoyl group can rotate freely in the complex as is indicated by a rotational correlation time of 0.2–1.0 ns [12]. Also a correlation time of more than 50 ns was found, which was attributed to the rotation of the whole complex (expected correlation time 2 µs). When using the triplet probe cosinemaleimide only mobility of the whole complex was found, showing that the label is folded back to the protein surface [13]. From a lack in energy transfer between the lipoyl domain and the FAD group of E₃ it is suggested that the lipoyllysine residue is too short to serve all catalytic centres [14] and

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therefore at least a part of the protein chain around the lipoic residue is thought to be flexible. Direct evidence for conformational flexibility in E2 chains was obtained from \(^1\)H-NMR spectroscopy [15]. In these experiments the regions, which are very rich in alanyl and prolyl residues, are shown to possess conformational flexibility. Thus, from these experiments it has been suggested that all three subdomains in the lipoic domain can rotate independently. In this paper it is shown with fluorescence anisotropy decay experiments that, although the alanine-proline rich region may possess internal flexibility, the lipoic domain moves as a single entity within the large multimeric E2 core. This movement is restricted by the binding of the peripheral components.

2. MATERIALS AND METHODS

2-(4-Iodoacetamidoanilino)-naphthalene-6-sulfonic acid (IAANS) was obtained from Molecular Probes. Dihydrolipoyl transacetylase (Ez) was isolated from the pyruvate dehydrogenase complex by covalent chromatography on thiol-Sepharose 4B as described previously [16], with modifications according to [7]. The lipoic domain was obtained after limited proteolysis with trypsin of the Ez component, which was covalently bound on thiol-Sepharose 4B and purified as described before [7]. Labelling was carried out as follows. Ez and the lipoic domain were incubated for 30 min at 4°C in 20 mM Tricine, pH 8.5, containing 20 mM dithiothreitol, and anaerobically dialysed against standard buffer (50 mM potassium phosphate, pH 7.0, containing 0.5 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride). From a 15.9 mM IAANS stock solution in \(\text{H}_2\text{O}/\text{ethanol (1:1, v/v)}\) a fifteen times molar excess to sulfhydryl groups of the lipoic moieties was added, and after 30 min the sample was dialysed against standard buffer. From optical density measurements it was observed that over 80% of the lipoic groups were (doubly) labelled. Since it has been shown [12] that only 2-2.5 of the three potential sites per Ez chain contain a lipoic group, it is clear that all present lipoic groups were (doubly) labelled. No bound label was detected without prior reduction. After labelling the complex activity had totally disappeared, but the Ez (transacetylase) activity was fully retained. Fluorescence spectra were recorded on an Aminco SPF-500 fluorimeter. Time-resolved fluorescence decay was measured with a system consisting of a frequency-doubled synchronously pumped dye laser for excitation and time-correlated single photon counting in detection. The excitation wavelength was at 310 nm and the emission was monitored via a 450 nm band-pass filter (Balzers K45). Details of the experimental set-up have been described in [17]. All experiments were carried out at 20°C. Data analysis was performed as described in [18]. The order parameter \(S_1\) is obtained from the relation \((S_1)^2 = \frac{1}{2}\cos^2(\theta_1(1 + \cos\theta_1)).\)

3. RESULTS AND DISCUSSION

The initial fluorescence anisotropy decay curves are shown in figs 1 and 2. Fig.1 shows the experimental and the fitted curve of the fluorescence anisotropy decay of Ez, in fig.2 only the fitted curves of the lipoic domain, E2, and E2 with bound Ez and E3 components are shown. For the lipoic domain the anisotropy decays as a double exponential function with a short (0.6 ns) and a longer (11.4 ns) characteristic time constant. The short component can be ascribed to the motion of the lipoic group rotating freely around the linkage with the protein chain. This time constant correlates well with the values found previously with spin labels [12]. The longer component can be ascribed to the rotation of the whole domain. The correlation time can be calculated on the basis of an empirical formula relating the correlation time

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\text{Fig.1. Fluorescence anisotropy decay of Ez. Shown are two curves: the experimental (noisy curve) and the calculated (smooth curve) fluorescence anisotropy decay. The parameters of the triple exponential decay are listed in table 1. The quality of the fit is indicated by the weighted residuals and the autocorrelation function, shown in the upper panels. The statistical parameters are } \chi^2 = 1.06, \text{ and the Durbin-Watson parameter } = 2.03.
\]
Fig. 2. Fluorescence anisotropy decay of the lipoyl domain, $E_2$, and $E_2$ complexed with $E_1$ or $E_3$. Shown are the fitted curves of (1) the lipoyl domain ($\circ$), (2) $E_2$ (O), (3) $E_2 + E_3$ (A) in a 4:2 molar concentration, when the $E_3$ binding site is saturated by $E_3$, (4) $E_2 + E_3$ (A) in a 4:8 molar concentration, when both the $E_1$ and $E_2$ binding sites are saturated by $E_1$, and (5) $E_2 + E_1$ (O) in a 4:6 molar concentration, when the $E_1$ binding sites are saturated by $E_1$. The parameters of the double (1) and the triple (2–5) exponential decays and the standard errors derived from the fits are listed in Table 1.

φ with $M_t$ of a hydrated, spherical polypeptide at 20°C: $\phi(n)=3.84 \times 10^{-4} \cdot M_t$ [20]. From this formula a correlation time of about 12.5 ns is calculated for the lipoyl domain having an $M_t$ value of 32600 [7]. Despite the fact that the lipoyl domain is thought to have a swollen or extended structure, $f/f_0 = 1.7$ [7], the agreement is rather good. For $E_2$ the anisotropy decay curve can be described as a triple exponential function with a short (0.4 ns), a longer (10.7 ns) and a very long (600 ns) time constant. The latter is fixed in the fitting procedure and represents the rotation of the whole protein (1.6 MDa). The short component is in the same order as found in the lipoyl domain and accounts for the free rotation of the lipoyl group. The time constant of 10.7 ns will represent the rotation of the lipoyl domain, which is thought to rotate independently of the large $E_2$ core. From the order parameter $S_1$ and the related cone angle $\theta_c$ that can be derived from a wobbling-in-cone model [19] it is shown that in $E_2$, where the lipoyl domain is bound to the core, the order increases and the motion of the label is more restricted. This indicates that the motion of the lipoyl domain is not totally independent of the core, but a certain degree of interaction exists. Upon binding of $E_1$ or $E_3$ components to the $E_2$ core no significant difference in correlation times is found (Table 1, expts 3–5). From $\beta_3$, $S_1$ and $S_2$ it is clear that, despite the dissociation of $E_2$ which takes place upon addition of $E_1$ (or excess $E_3$), a significant difference is found in order and in motional restriction upon binding of the $E_1$ component (expt 5). Upon addition of excess $E_3$ (expt 4), which is thought to bind on or near the $E_1$ binding site [1] no significant difference is observed. Obviously some interaction exists between $E_1$ and the lipoyl domain which is not present between $E_3$ and the lipoyl domain.

<table>
<thead>
<tr>
<th>Expt</th>
<th>Protein</th>
<th>$M_t$</th>
<th>$\beta_1$</th>
<th>$\phi_1$</th>
<th>$\beta_2$</th>
<th>$\phi_2$</th>
<th>$\beta_3$</th>
<th>$\phi^b$</th>
<th>$S_1^c$</th>
<th>$\theta^d$</th>
<th>$S_2^e$</th>
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<tr>
<td>1</td>
<td>Lipoyl domain</td>
<td>33000</td>
<td>0.160 ± 0.011</td>
<td>0.62 ± 0.19</td>
<td>0.039 ± 0.002</td>
<td>11.4 ± 1.5</td>
<td>-</td>
<td>-</td>
<td>0.44 ± 0.03</td>
<td>56 ± 2</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>$E_2$</td>
<td>156000</td>
<td>0.069 ± 0.002</td>
<td>0.38 ± 0.07</td>
<td>0.048 ± 0.002</td>
<td>10.7 ± 1.4</td>
<td>0.024 ± 0.003</td>
<td>600 ± 0.74</td>
<td>± 0.04</td>
<td>36 ± 3</td>
<td>0.57 ± 0.03</td>
</tr>
<tr>
<td>3</td>
<td>$E_2 + E_1$ (4:2)</td>
<td>216000</td>
<td>0.042 ± 0.004</td>
<td>0.91 ± 0.13</td>
<td>0.051 ± 0.006</td>
<td>18.1 ± 3.7</td>
<td>0.025 ± 0.006</td>
<td>830 ± 0.80</td>
<td>± 0.11</td>
<td>31 ± 8</td>
<td>0.57 ± 0.11</td>
</tr>
<tr>
<td>4</td>
<td>$E_2 + E_3$ (4:8)</td>
<td>660000</td>
<td>0.055 ± 0.008</td>
<td>0.56 ± 0.08</td>
<td>0.060 ± 0.005</td>
<td>16.9 ± 2.5</td>
<td>0.026 ± 0.005</td>
<td>253 ± 0.78</td>
<td>± 0.10</td>
<td>32 ± 8</td>
<td>0.55 ± 0.08</td>
</tr>
<tr>
<td>5</td>
<td>$E_2 + E_3$ (4:6)</td>
<td>860000</td>
<td>0.041 ± 0.004</td>
<td>1.00 ± 0.14</td>
<td>0.047 ± 0.006</td>
<td>20.0 ± 4.7</td>
<td>0.055 ± 0.007</td>
<td>330 ± 0.84</td>
<td>± 0.10</td>
<td>27 ± 9</td>
<td>0.73 ± 0.09</td>
</tr>
</tbody>
</table>

* See legend of fig.2 for details

* Fixed in the analysis

* From $(S_1)^2 = \frac{\beta_2 + \beta_3}{\beta_1 + \beta_2 + \beta_3}$

* From $(S_3)^2 = \frac{\theta_1}{\beta_1 + \beta_3}$

* From $(S_2)^2 = \frac{\beta_3}{\beta_2 + \beta_3}$

Table 1: Fluorescence decay parameters of the lipoyl domain, $E_2$ and $E_2$ complexed with $E_3$ or $E_1$.
could be related to the observation of Packman et al. [8] who showed that E₃ is able to use lipoamide as a substrate, whereas E₁ needs the intact lipoyl subdomain as substrate. Mobility of lipoyl subdomains has been suggested from ³¹H-NMR experiments, based on observed conformational mobility of the alanine and proline rich regions. In these fluorescence anisotropy experiments we present a direct indication that the lipoyl domain as a whole contains mobility more or less independent of the large E₂ core. The hinge of this mobility should be located between the lipoyl domain and the catalytic domain (residue 331–381 [6]). In this region also the binding sites for the E₁ and E₃ components are located [7]. After binding of these components to the E₂ core no dramatic limitation of mobility is observed, suggesting that the hinge of the mobility of the lipoyl domain is located N-terminal of the E₁ and E₃ binding sites. Previous fluorescence anisotropy experiments of the FAD in free and in bound E₃ have shown that E₃, bound to the E₂ core, still possesses a high mobility [21]. This indicates a second hinge C-terminal to the E₃ binding site. This region may correspond with a highly mobile region observed in ¹H-NMR experiments near the N-terminus of the catalytic domain [22].

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