Volume 240, number 1,2, 205-210 FEB 06524 November 1988

Mobile sequences in the pyruvate dehydrogenase complex, the E_2 component, the catalytic domain and the 2-oxoglutarate dehydrogenase complex of *Azotobacter vinelandii*, as detected by 600 MHz ^{1}H-NMR spectroscopy

Roeland Hanemaaijer, Jacques Vervoort, Adrie H. Westphal, Arie de Kok and Cees Veeger

Department of Biochemistry, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

Received 26 September 1988

600 MHz ^{1}H-NMR spectroscopy demonstrates that the pyruvate dehydrogenase complex of *Azotobacter vinelandii* contains regions of the polypeptide chain with intramolecular mobility. This mobility is located in the E_2 component and can probably be ascribed to alanine-proline-rich regions that link the lipoyl subdomains to each other as well as to the E_1 and E_3 binding domain. In the catalytic domain of E_2, which is thought to form a compact, rigid core, also conformational flexibility is observed. It is conceivable that the N-terminal region of the catalytic domain, which contains many alanine residues, is responsible for the observed mobility. In the low-field region of the ^{1}H-NMR spectrum of E_2 specific resonances are found, which can be ascribed to mobile phenylalanine, histidine and/or tyrosine residues which are located in the E_1 and E_3 binding domain that links the lipoyl domain to the catalytic domain. In the ^{1}H-NMR spectrum of the intact complex, these resonances cannot be observed, indicating a decreased mobility of the E_1 and E_3 binding domain.

Dihydrolipoyl transacetylase; Pyruvate dehydrogenase complex; ^{1}H-NMR spectroscopy; Mobility; (*Azotobacter vinelandii*)

1. INTRODUCTION

The pyruvate dehydrogenase complex catalyzes the following reaction:

$$\text{Pyruvate} + \text{CoA} + \text{NAD}^+ \rightarrow \text{AcetylCoA} + \text{CO}_2 + \text{NADH} + \text{H}^+$$

It is composed of multiple copies of three enzymes: pyruvate dehydrogenase (E_1), dihydrolipoyl transacetylase (E_2) and lipoamide dehydrogenase (E_3). The E_2 component forms a structural core to which E_1 and E_3 components are bound non-covalently. During catalysis the substrate is carried in a thioester linkage by lipoyl-lysine residues that are part of the E_2 component and act as 'swinging arms' while transferring the substrates, acetyl groups and reduction equivalents, between the active sites of the different components [1].

The domain structure of E_2 has been examined by limited proteolysis [2]. After digestion of E_2 with trypsin a lipoyl domain and a catalytic domain are obtained. The lipoyl domain carries the lipoyl groups. The catalytic domain possesses the transacetylase active site and the E_2 intersubunit-binding sites, showing the same 24-meric structure as intact E_2 [3]. The E_1- and E_3-binding sites are lost during proteolysis. They are thought to be located on a ± 6 kDa region that links the catalytic domain to the lipoyl domain.

The gene encoding E_2 of the pyruvate dehydrogenase complex of *Azotobacter vinelandii* has been cloned and sequenced [4]. From the amino acid sequence derived from the DNA sequence it is shown that the lipoyl domain accounts for the N-terminal half of the polypeptide chain and comprises three highly homologous structures, each carrying a potential lipoyl-binding site. In *Escherichia coli* it

Correspondence address: A. de Kok, Dept of Biochemistry, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

Published by Elsevier Science Publishers B.V. (Biomedical Division)

is shown that these repeats exist as three independent folded entities that retain their function as substrate for reductive acetylation by the E_1 component [5]. They are linked to each other and to the E_1- and E_3-binding domain by means of regions ($\pm$ 20–30 residues) that are very rich in analine and proline residues [4,6].

The active sites of the different components are at least 4 nm apart in the complex, too far away for a single lipoyl-lysine group [7,8]. Therefore it has been suggested that the lipoyl domain might be sufficiently flexible to facilitate movement of the lipoyl group between the different active sites. Strong evidence in favor of the existence of conformationally flexible regions in 2-oxoacid dehydrogenase complexes from *E. coli* [9,10], *Bacillus staerothermophilus* [11,12] and ox heart [13] was obtained by using ^{1}H-NMR spectroscopy. Unusual sharp resonances in the 270 and 400 MHz ^{1}H-NMR spectra of the intact complexes are thought to originate from alanine-proline-rich regions in the E_2 chains. In *E. coli* it has been suggested that the mobility of these regions is responsible for the independent mobility of the three lipoyl subdomains [14,15]. Mobility of the lipoyl domain has also been detected by time-resolved fluorescence spectroscopy [16]. After labelling of the lipoyl groups of E_2 from *A. vinelandii* with a fluorophore, mobility is observed of the labelled lipoyl group, of the lipoyl domain and of the whole complex. No independent mobility of the three lipoyl subdomains has been observed.

After cloning in *E. coli*, a high expression of the gene encoding E_2 was obtained [17]. Both E_2 and the catalytic domain, obtained by limited proteolysis, showed to be soluble at high protein concentrations and were used in 600 MHz ^{1}H-NMR experiments.

2. MATERIALS AND METHODS

2.1. *Materials*

Tosylphenylalanylchloromethane-treated trypsin was obtained from Worthington and phenylmethylsulfonyl fluoride was from Sigma. Deuterium oxide (99.8%) was purchased from Ega-chemie.

2.2. *Isolation of the proteins*

The pyruvate dehydrogenase complex was isolated from *Azotobacter vinelandii* as described by Bosma et al. [18]. The 2-oxoglutarate dehydrogenase complex was isolated from *A. vinelandii* as described in [19]. The E_2 component of the *A. vinelandii* pyruvate dehydrogenase complex was isolated from *Escherichia coli*, in which the gene encoding *A. vinelandii* E_2 was cloned [17]. The catalytic domain, obtained by limited proteolysis of *A. vinelandii* E_2 with trypsin, was isolated by gel chromatography, using FPLC equipped with a Superose 6 K26/70 column (Pharmacia), and concentrated by ultrafiltration using an Amicon YM-100 membrane.

2.3. *^{1}H-NMR spectroscopy*

Protein samples for ^{1}H-NMR spectroscopy (in standard buffer: 50 mM potassium phosphate, pH 7.0, containing 0.1 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride) were concentrated to about 0.5–1.0 mM. ^2H_2O was added to a final concentration of 10% (v/v). ^{1}H-NMR spectra were obtained at 600 MHz with a Bruker AM-600 spectrometer using a 16.7 kHz spectral width, 30° pulses and a repetition time of 1 s. 8K data points were used. Quadrature detection and quadrature phase cycling were used. Chemical shifts are relative to trimethylsilylpropionate (TSP). Wilmad 5 mm NMR-tubes were used. The solvent resonance irradiation (18 dB attenuation from 0.2 W) was applied at all times, except during data acquisition. The transmitter frequency was placed exactly at the waterresonance. The sample temperature was 23°C. The NMR data were transferred to a MicroVAX II and evaluated with (modified) software, kindly given to us by Dr R. Boelens. The FIDs were zero-filled once before Fourier transformation (without any window function). The resulting frequency spectrum was manipulated by a fourth order polynome for baseline correction.

2.4. *Other techniques*

Protein concentrations were measured according to Lowry et al. [20]. A stock solution of 1 M phenylmethylsulfonyl fluoride was prepared in $C^2H_3O^2H$ and diluted 100-fold in standard buffer.

3. RESULTS AND DISCUSSION

3.1. *High-field resonances*

In ^{1}H-NMR spectra of large protein complexes (MDa) in general no distinct resonances can be observed; the calculated linewidth of a methylene proton resonance is in the range of kHz. However, in the 600 MHz spectrum of the pyruvate dehydrogenase complex (fig.1A), superimposed on the expected broad component in the spectrum, many sharp resonances are observed (linewidth in the range of 10–100 Hz). These resonances must arise from a region or regions of the polypeptide chain having substantial mobility. The major sharp resonance occurs at 1.39 ppm which, on the basis of the chemical shift, can be ascribed to the methyl side chains of alanine residues. In the intact pyruvate dehydrogenase complex of *E. coli* a similar sharp resonance has been found [9, 10]. It was ascribed to the three alanine-proline-rich

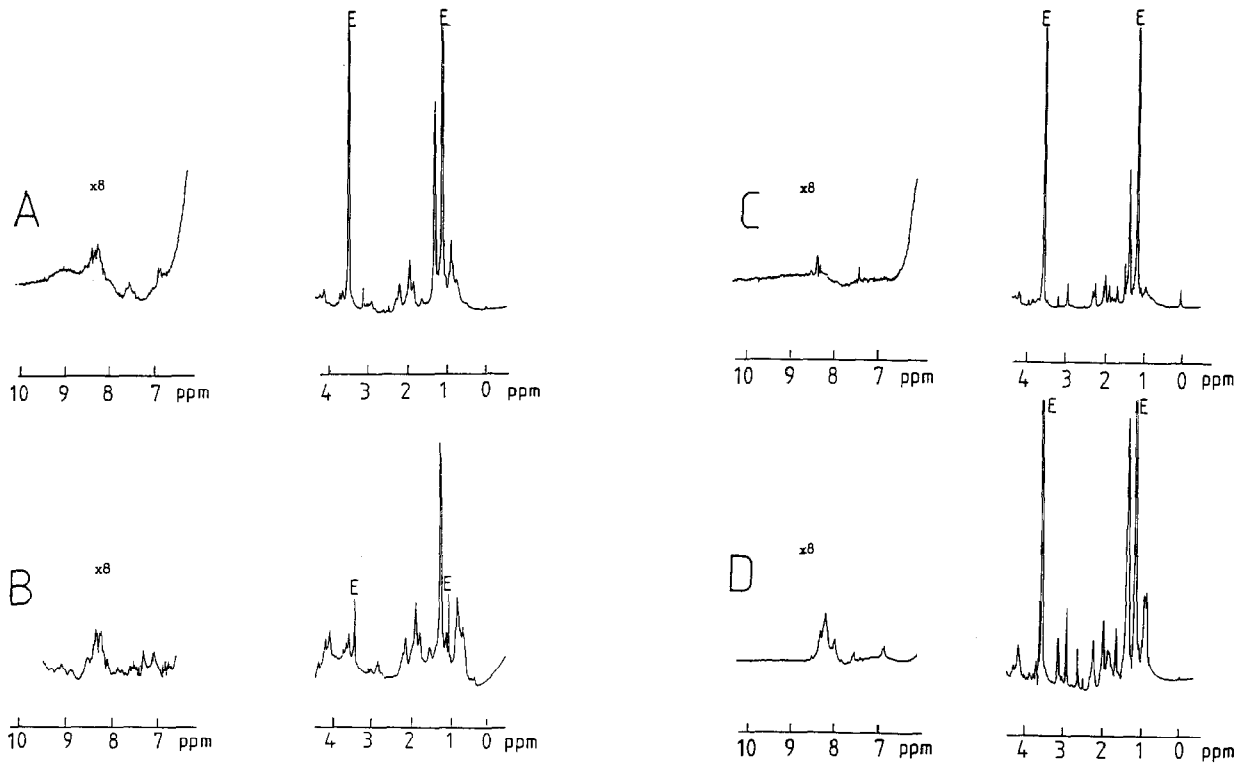

Fig.1. 600 MHz ^{1}H-NMR spectra of the pyruvate dehydrogenase complex, the E_2 component, the catalytic domain and the 2-oxoglutarate dehydrogenase complex from *A. vinelandii*. (A) Pyruvate dehydrogenase complex, 34 mg/ml (= 159 μM E_2); (B) E_2 component of the pyruvate dehydrogenase complex, 43.6 mg/ml (= 770 μM E_2); (C) catalytic domain of E_2, 23.7 mg/ml (= 862 μM); (D) 2-oxoglutarate dehydrogenase complex, 66.7 mg/ml (= 550 μM E_2). The sharp resonances marked E are from ethanol.

regions (20–30 residues each) of the E_2 component, located at the links between the lipoyl subdomains and between the lipoyl domain and the E_1- and E_3-binding domain. In contrast to experiments with the *E. coli* complex [9], we were able to isolate E_2 and concentrate it up to 1 mM, without aggregation of the protein. Indeed, the 600 MHz ^{1}H-spectrum of E_2 (fig.1B) showed similar sharp resonances, which is direct evidence that the mobile regions are located on the E_2 chain. Also many sharp resonances were found between 1.5 and 2.5 ppm, which can partly be ascribed to mobile proline residues present in the same regions. In the high-field spectrum of OGDC (fig.1D), a similar pattern of sharp resonances is observed, suggesting a similar mobile region, probably an alanine-proline-rich region which has been found in the E_2 sequence of *A. vinelandii* OGDC, located between its single lipoyl domain and the proposed E_1- and E_3-binding domain (unpublished).

Two additional resonances of ethanol can be observed, which are due to a contamination. The concentration of ethanol is estimated to be around 5–10 mM.

Our results are in agreement with the proposal that the conformational mobility can be ascribed to the alanine-proline-rich regions, which is confirmed by experiments with genetic engineered *E. coli* complexes, in which mutations or deletions have been introduced in the alanine-proline-rich regions [14,15]. A further confirmation came from experiments using a synthetic alanine-proline-rich polypeptide, representing one of these regions in *E. coli* E_2 [21].

From the NMR experiments on the *E. coli* complex it has been suggested that the presence of the highly mobile alanine-proline sequences gives an independent conformational mobility to the three repeating subdomains in the lipoyl domain [14,15]. However, no sharp resonances of the subdomains

(±8 kDa) are observed in the spectra of the whole complex. Similar results have been obtained from NMR spectra of OGDC of *Bacillus staerothermophilus* [12]. This complex contains only one lipoyl domain per E_2 chain. This lipoyl domain contains one tryptophan, one tyrosine and four phenylalanine residues. Since no aromatic proton resonances of the lipoyl domain were visible in the spectrum of the intact complex, it has been suggested that the lipoyl domain is not free to move rapidly, but must spend a major part of the time bound to one of the other components of the complex. Thus, a mobile alanine-proline region can be observed without the corresponding mobility of the lipoyl domain. In the spectra of *A. vinelandii* PDC, OGDC or E_2 no indications for a small, mobile lipoyl subdomain are found. This is in agreement with results obtained from fluorescence anisotropy experiments in which mobility of the single lipoyl-label group, the whole complex and the whole lipoyl domain (32 kDa) was found, but no mobility of a lipoyl subdomain (9 kDa) could be observed. Obviously, using ^{1}H-NMR spectroscopy, no conclusions can be drawn about mobility of the lipoyl subdomains, when only mobility of the alanine-proline-rich region is observed.

3.2. *Low-field resonances in E_2*

In the aromatic region of the E_2 spectrum (fig.1B), two specific sharp resonances (7.1 and 7.3 ppm) are observed which are not present in the spectrum of PDC (fig.1A) or in that of the catalytic domain (fig.1C). The intensity of these residues is about 3% of the alanine methyl resonance, which indicates that they can be assigned to either one or two aromatic residues, i.e. to phenylalanine, histidine or tyrosine. These amino acid residues are not present in the lipoyl domain of E_2 (fig.2) [4]. Most of the aromatic amino acid residues are located in the catalytic domain but obviously not in a mobile region since in the spectrum of the catalytic domain no sharp resonances are observed which can be ascribed to these residues, although sharp resonances are present at low field (fig.1C). However, both a histidine, a phenylalanine and a tyrosine residue are present in the region located between the lipoyl and the catalytic domain, possessing the E_1- and E_3-binding sites. Obviously, this region possesses conformational mobility. This is in agreement with previous results from fluorescence anisotropy of FAD in free and bound E_3. After binding of E_3 to the E_2-core almost no change in rotational correlation coefficient was observed, from which it was suggested that the binding region should possess mobility [22].

No sharp resonances of residues from this region are observed in the spectrum of the whole complex. Obviously after binding of the large E_1 dimer (200 kDa) and E_3 dimer (100 kDa), the mobility of this region in E_2 diminishes to an extent which cannot be observed anymore as a sharp resonance by ^{1}H-NMR spectroscopy. In *E. coli* MNR spectra, mobility of residues in this region has never been observed since E_2 of *E. coli* PDC aggregates at the high protein concentrations needed for NMR experiments [9].

3.3. *Mobility in the catalytic domain*

The catalytic domain is thought to form a compact rigid core (0.5–0.6 kDa) [2]. However, also in the spectrum of this domain sharp resonances are observed (fig.1C). The most prominent, sharp resonance is at 1.39 ppm; the methyl resonance of alanine residues. The linewidth of this peak is less than that observed in the spectra of PDC, E_2 and OGDC. Its intensity, however, is much less than in the other spectra. A second sharp resonance is found very close to it at 1.50 ppm. This resonance cannot be observed in PDC and OGDC. Probably both resonances can be attributed to a region rich in alanine residues located at the N-terminus of the catalytic domain. In the spectrum of E_2 it is partly masked by the large resonance at 1.39 ppm. Probably the alanine residues, located at the N-terminus of the catalytic domain, become more mobile when the N-terminal half of the E_2 chain is excised after proteolysis, or they can partly change to a somewhat different environment. In E_2 and the catalytic domain, the environment can differ from that in the whole complex, since no E_1- and E_3 components are bound, and the uncomplexed-binding domain possesses mobility. A mobile region in the catalytic domain has also been observed in *E. coli* PDC. A sharp resonance at 1.52 ppm was observed which was not present in the whole complex. It was ascribed to a very small alanine-rich region at the N-terminus of the catalytic domain. In the spectrum of the catalytic domain of OGDC E_2 from *Bacillus staerothermophilus* [12] a small resonance at 1.36 ppm was observed, but was ascribed (may-

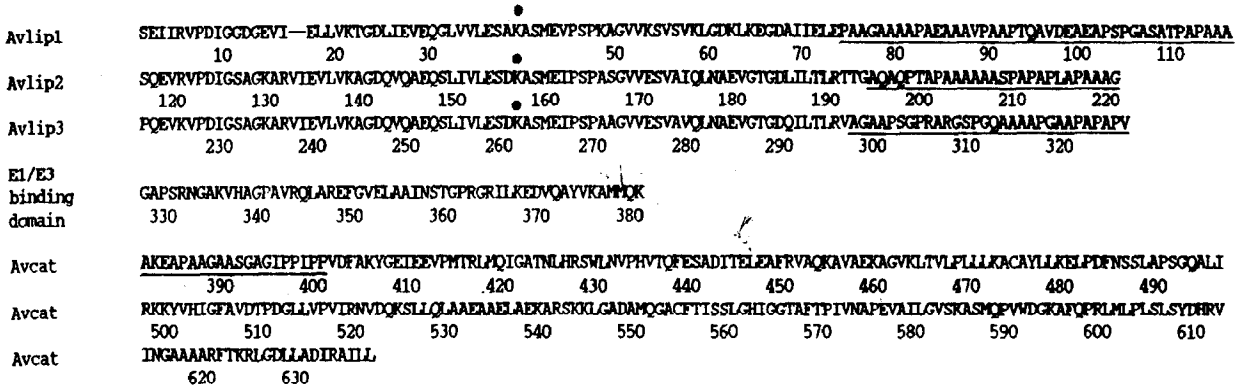

Fig.2. Primary structure of the E_2 chain of the pyruvate dehydrogenase complex from *A. vinelandii*. The proposed lipoyl-binding sites are indicated by (●). The alanine-proline-rich regions are underlined. lip, lipoyl domain; cat, catalytic domain.

be by the lack of the amino acid sequence) to a non-protein contaminant. Recently a part of the amino acid sequence of this protein has been published and a small alanine-rich region is shown at the N-terminus of the catalytic domain [23].

The spectrum of the catalytic domain of E_2 from *A. vinelandii* shows sharper resonances than those found in the spectra of PDC and E_2. This can probably be explained by the smaller region that is mobile. Although a limited amount of sharp resonances is present in the spectrum of the catalytic domain, these resonances cannot exclusively be ascribed to alanine residues. In addition to the 1.39 ppm and the 1.50 ppm resonances in the high-field region (0–4.5 ppm), some resonances are observed which can probably be ascribed to amino acid residues present in the N-terminal region of the catalytic domain (e.g. prolines, lysine and isoleucines). Also in the low-field region (7.5–8.5 ppm), a few resonances are observed which can probably be ascribed to resonances from amide protons that are protected from water, suggesting that part of the mobile region has a folded structure [24]. The resonance at 7.45 ppm can be ascribed to phenylmethylsulfonyl fluoride, which is added during isolation of the catalytic domain.

Acknowledgements: We would like to thank Mr C. van Mierlo for recording some NMR spectra. This investigation was supported by the Netherlands Foundation for Chemical Research (SON), with the financial aid from the Netherlands Organisation for Scientific Research (NWO).

REFERENCES

[1] Koike, M., Reed, L.J. and Caroll, W.R. (1963) J. Biol. Chem. 238, 30–39.
[2] Hanemaaijer, R., De Kok, A., Jolles, J. and Veeger, C. (1987) Eur. J. Biochem. 169, 245–252.
[3] Hanemaaijer, R., Westphal, A.H., Van de Heiden, T., De Kok, A. and Veeger, C. Eur. J. Biochem., submitted.
[4] Hanemaaijer, R., Janssen, A., De Kok, A. and Veeger, C. (1988) Eur. J. Biochem. 174, 593–599.
[5] Packman, L.C., Hale, G. and Perham, R.N. (1984) EMBO J. 3, 1315–1319.
[6] Stephens, P.E., Darlison, M.G., Lewis, H.M. and Guest, J.R. (1983) Eur. J. Biochem. 133, 481–489.
[7] Shepherd, G.B. and Hammes, G.G. (1976) Biochemistry 16, 5234–5241.
[8] Scouten, W.H., De Graaf-Hess, A.C., De Kok, A., Visser, A.J.W.G. and Veeger, C. (1978) Eur. J. Biochem. 84, 17–25.
[9] Perham, R.N., Duckworth, H.W. and Roberts, G.C.K. (1981) Nature 292, 474–477.
[10] Roberts, G.C.K., Duckworth, H.W., Packman, L.C. and Perham, R.N. (1983) Ciba Symp. 93, 47–62.
[11] Duckworth, H.W., Jaenicke, R., Perham, R.N., Wilkie, A.O.M., Finch, J.T. and Roberts, G.C.K. (1982) Eur. J. Biochem. 124, 63–69.
[12] Packman, L.C., Perham, R.N. and Roberts, G.C.K. (1984) Biochem. J. 217, 219–227.
[13] Wawrzynczak, E.J., Perham, R.N. and Roberts, G.C.K. (1981) FEBS Lett. 131, 151–154.
[14] Radford, S.E., Laue, E.D., Perham, R.N., Miles, J.S. and Guest, J.R. (1987) Biochem. J. 247, 641–649.
[15] Texter, F.L., Radford, S.E., Laue, D.E., Perham, R.N., Miles, J.S. and Guest, J.R. (1988) Biochemistry 27, 289–296.
[16] Hanemaaijer, R., Masurel, R., Visser, A.J.W.G., De Kok, A. and Veeger, C. (1988) FEBS Lett., in press.
[17] Hanemaaijer, R., Westphal, A.H., Berg, A., De Kok, A. and Veeger, C. Eur. J. Biochem., submitted.

[18] Bosma, H.J., De Graaf-Hess, A.C., De Kok, A., Veeger, C., Visser, A.J.W.G. and Voordouw, G. (1982) Ann. NY Acad. Sci. 378, 265–285.
[19] Bosma, H.J. (1984) Ph.D. Thesis, Wageningen.
[20] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[21] Radford, S.E., Laue, E.D. and Perham, R.N. (1986) Biochem. Soc. Trans. 14, 1231–1232.
[22] Grande, H.J., Visser, A.J.W.G. and Veeger, C. (1980) Eur. J. Biochem. 106, 361–369.
[23] Packman, L.C., Borges, A. and Perham, R.N. (1988) Biochem. J. 252, 79–86.
[24] Wüthrich, K. (1986) NMR of Proteins and Nucleic Acids, John Wiley & Son, New York.