PYRUVATE DEHYDROGENASE MULTIENZYME COMPLEX OF *ESCHERICHIA COLI*

Determination of the M_r of the lipoate acetyltransferase component

Michael J. DANSON and Caroline E. PORTEOUS
Department of Biochemistry 4 West, University of Bath, Claverton Down, Bath, BA2 7AY, England

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1. Introduction

Lipoate acetyltransferase (E2) forms the structural core of the pyruvate dehydrogenase multienzyme complex of *Escherichia coli*. It comprises 24 polypeptide chains [1–3] to which are bound multiple copies of the other two component enzymes, pyruvate decarboxylase (E1) and lipoamide dehydrogenase (E3) (reviewed [1,4]). There is a continuing controversy over the stoicheiometry of polypeptide chains in the native complex. Direct quantitation of the polypeptides separated by SDS–polyacrylamide gel electrophoresis indicates stoicheiometries of ~1.3:1:1 (E1:E2:E3) in complex as normally isolated [5–7] with an upper limit of 2:1:1 [8,9]. On the other hand, calculations by an indirect method, from the M_r of the complex and of the E2 core, gave a polypeptide stoicheiometry of 1:1:0.5 [10,11].

Inherent in this discrepancy is a disagreement over the M_r of the E2 polypeptide. From a detailed study using SDS–polyacrylamide gel electrophoresis a value of 83 000 was obtained [12]. However, it was claimed in [13] that the polypeptide may migrate anomalously on electrophoresis due to its acidic nature; using sedimentation equilibrium analysis in 6 M guanidine–HCl they report the M_r of E2 to be 60 000–64 000. In [11] M_r values between these two were found but reduction and alkylation of E2 reduced it to $M_r \sim 35\,000$.

In view of the need for the precise value of E2 polypeptide M_r in calculation of stoicheiometries we thought it essential to re-examine the problem. Gel filtration in guanidine–HCl was chosen as it is a method for which anomalous behaviour of proteins has not yet been reported [14,15] and it is one which has resolved a similar discrepancy in the mammalian pyruvate dehydrogenase complex [15].

Abbreviations: E1, pyruvate decarboxylase (EC 1.2.4.1); E2, lipoate acetyltransferase (EC 2.3.1.12); E3, lipoamide dehydrogenase (EC 1.6.4.3); M_r, relative molecular mass; SDS, sodium dodecylsulphate

2. Experimental

2.1. *Proteins and reagents*

Pyruvate dehydrogenase complex was purified from a mutant of *E. coli* K12 constitutive for production of the complex [2]. Cytochrome *c* (equine), glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), aldolase (rabbit muscle), serum albumin (bovine), phosphorylase *a* (rabbit muscle) and β-galactosidase (*E. coli*) were from Boehringer Mannheim. Sephacryl S-300 and blue dextran-2000 were from Pharmacia, Uppsala. Guanidine–HCl was from Fluka AG, Buchs, and was purified by millipore filtration after treatment of a 6 M solution with activated charcoal. Iodo-[2-^{14}C]acetic acid (5 Ci/mol) and [2-^{14}C]pyruvate (16 Ci/mol) were from the Radiochemical Centre, Amersham. All other chemicals were of Analar grade.

2.2. *Methods*

The 3 polypeptide chains of pyruvate dehydrogenase complex and the calibrating proteins were radiolabelled with iodo-[2-^{14}C]acetic acid in 6 M guanidine–HCl according to [16]. Specific radiolabelling of E2 was achieved by reaction of native complex with *N*-ethylmaleimide in the presence of [2-^{14}C]-pyruvate [17] followed by dilution into 6 M guanidine–HCl.

Gel filtration on Sephacryl S-300 (column dimensions: 1.6 × 76 cm) was carried out according to

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[16]. The elution buffer was 6 M guanidine–HCl (pH 5.0) and the column was operated at a flow rate of 5 ml/h; 0.5 ml fractions were collected. Blue dextran and 2-nitro-5-mercaptobenzoate were used as markers for the void and total volume, respectively, and were assayed, spectrophotometrically at 630 nm and 412 nm. Eluted polypeptides were detected by counting samples of each fraction for radioactivity. From the elution volumes for each protein, values of K_d, the distribution coefficient, were calculated according to [18]:

$$K_d = (V_e - V_o)/(V_{re} - V_o)$$

where V_e, V_o and V_{re} represent the elution volume of the protein, blue dextran and 2-nitro-5-mercaptobenzoate, respectively.

SDS–polyacrylamide gel electrophoresis was done as in [19]. Radioactivity in the stained protein bands was measured as in [17].

3. Results

Electrophoresis on SDS–polyacrylamide gels showed that each of the standard proteins (cytochrome *c*, M_r 12 500; glyceraldehyde-3-phosphate dehydrogenase, M_r 36 000; aldolase, M_r 39 000; serum albumin, M_r 66 300; phosphorylase *a*, M_r 97 400 and β-galactosidase, M_r 116 000) were homogenous and that reduction and *S*-carboxymethylation did not affect their electrophoretic mobilities. This was also shown to be the case for the 3 polypeptide chains (E1, E2, E3) of the pyruvate dehydrogenase complex, and with reference to the standard proteins, app. M_r-values of 99 000 (E1), 81 000 (E2) and 53 000 (E3) were calculated. These values correspond very closely with those reported in SDS gel electrophoresis [10,12,19,20]. The specific radiolabelling of E2 with [2-^{14}C]pyruvate (see section 2) was checked by measurement of the radioactivity in the 3 protein bands after SDS gel electrophoresis. It was found that >90% of the radioactivity incorporated into the complex was located in the E2 component.

The data from the gel filtrations on Sephacryl S-300 are plotted in the form ln(100 . K_d) vs $N^{2/3}$ (fig.1) where N is the number of amino acid residues in the polypeptide chain [21]. A good fit to a straight line is observed for the standard proteins over 12 500–116 000 M_r (r^2 = 0.998). The data for the E1 and E3 polypeptides fit closely to the calibration

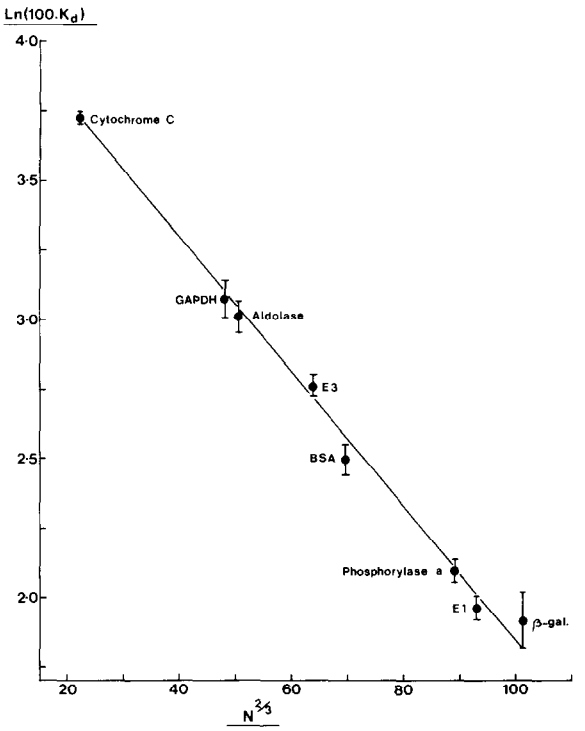

Fig.1. Gel filtration in 6 M guanidine–HCl. Plot of ln(100 . K_d) vs $N^{2/3}$ for the calibrating proteins and the E1 and E3 components of pyruvate dehydrogenase complex. N is the number of amino acid residues in the polypeptide chain. The following values of N were taken from published sequences: cytochrome *c*, 104; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 330; aldolase, 358; bovine serum albumin (BSA), 579; phosphorylase *a*, 841; β-galactosidase (β-gal.), 1021. Values of N for E1 and E3 are from published M_r-values and amino acid analyses: E1, 896; E3, 511; The data for E1 and E3 were not used in the regression analysis.

curve. This was expected as there is no disagreement over their M_r-values as determined by SDS gel electrophoresis, sedimentation analysis and light scattering measurements [10–12,19,20]. The elution position of E2 was the same whether from *S*-carboxymethylated complex or from complex specifically labelled with [2-^{14}C]pyruvate in the E2 component. The K_d-value obtained (0.094 ± 0.003) corresponds to 760 (±25) amino acids/E2 polypeptide chain. From the amino acid analysis of E2 [22] this gives an E2 of M_r 81 000 (±3000). This value is in excellent agreement with that obtained by SDS gel electrophoresis but differs considerably from the results of sedimentation equilibrium analysis [10,13].

After gel filtration, samples were taken from fractions containing the E1, E2 and E3 polypeptides and

were dialysed to remove the guanidine–HCl. On subsequent SDS gel electrophoresis, mobilities and app. M_r-values for each component were indistinguishable from the values obtained before *S*-carboxymethylation and gel filtration.

4. Discussion

These data fully support the results from SDS–polyacrylamide gel electrophoresis [10,12,19,20], indicating that the E2 polypeptide chain is ~80 000–83 000 M_r. This would imply that E2 does not migrate anomalously on electrophoresis despite its hydrophilic nature [13]. Indeed, from an analysis of the free electrophoretic mobilities of the pyruvate dehydrogenase component on SDS gels and their corresponding retardation coefficients, E1, E2 and E3 behaved normally as compared to standard proteins in this electrophoretic system [12]. However, this M_r is considerably greater than the M_r 60 000–64 000 determined by sedimentation analysis in 6 M guanidine–HCl [10,13]. An exact knowledge of $\bar{v}$ of E2 in the denaturant is required to obtain an accurate M_r-value from the ultracentrifugation data and it should be noted that in [13] $\bar{v}$ = 0.744 ml/g was taken, the value for native E2 calculated from its amino acid composition. However, differences in $\bar{v}$ as great as 0.03 ml/g have been observed between native proteins and their unfolded states in guanidine–HCl [23]. The value of $\bar{v}$ assumed for E2 may therefore be a serious source of error in the determination of M_r for the polypeptide [10,13].

We can find no evidence to support the observation in [11] that reduction and alkylation of E2 reduces the M_r to 33 000–36 000. On SDS gel electrophoresis and gel filtration in guanidine–HCl M_r ~ 81 000 was observed both before and after reduction and *S*-carboxymethylation. This indicates that the observed reduction of M_r [11] is due to enhanced proteolysis after alkylation rather than to disulphide cleavage.

An E2 M_r of 80 000–83 000 has been used in [2,6,7] in the determinations of polypeptide chain stoicheiometries in the pyruvate dehydrogenase complex. This confirmation of the M_r-value adds further weight to the validity of the conclusions that in native complex the chain ratio of E3:E2 approaches unity whereas there is a molar excess of the E1 component [2,6,7].

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