POLYPEPTIDE CHAIN STOICHEIOMETRY IN THE SELF-ASSEMBLY OF THE PYRUVATE DEHYDROGENASE MULTIENZYME COMPLEX OF *ESCHERICHIA COLI*

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1. Introduction

The pyruvate dehydrogenase multienzyme complex of *E. coli* contains three different types of polypeptide chain which are responsible for the three component enzymic activities. In order of their participation in the overall reaction, these are pyruvate decarboxylase (E1), lipoate acetyltransferase (E2) and lipoamide dehydrogenase (E3) (for reviews, see [1,2]). The lipoate acetyltransferase component forms a core to which E1 and E3 are bound and it is likely that E2 possesses 24 subunits arranged with octahedral symmetry. However, the total numbers of polypeptide chains of each type in the native enzyme are controversial. A stoicheiometry (E1 : E2 : E3) of 2:2:1 is favoured by Reed and co-workers [3] but other measurements of the stoicheiometry [4,5] have indicated values of $> 1:1:1$ and we have proposed [5,6] that a value of 2:1:1 may represent an upper limit.

On the other hand, it has been reported that there is probably steric hindrance in the binding of E1 and E3 such that the full capacity to bind 48 chains of E1 to the 24-chain core, E2, can only be realized in the absence of E3 and vice versa: the lipoate acetyltransferase was found to be able to accommodate a total of about 24 chains (12 dimers) of E1 and 12 chains (6 dimers) of E3 [7]. This corresponds with the chain stoicheiometry (E1 : E2 : E3) of 2:2:1 described previously [3].

To try to resolve these discrepancies, we have studied the self-assembly of the enzyme from the E1 component and the E2–E3 sub-complex at various pH values. We show that the E2–E3 sub-complex can bind up to 48 chains of E1 without apparent cooperativity and without apparent displacement of the E3 component and conclude that the limiting stoicheiometry of 2:1:1 proposed from earlier studies of native complex [5,6] is likely to be the value imposed by symmetry.

2. Materials and methods

2.1. *Enzymes and reagents*

The pyruvate dehydrogenase multienzyme complex was prepared from a constitutive mutant of *E. coli* by the method of Reed and Mukherjee [8]. Methyl [1-^{14}C]acetimidate was synthesized from [1-^{14}C]-acetonitrile (The Radiochemical Centre, Amersham, Bucks, UK) as described elsewhere [9]: the specific radioactivity was about 500 000 dpm/μmol. All other reagents were of AR grade and were used without further purification.

2.2. *Resolution and reconstitution of the enzyme complex*

The pyruvate dehydrogenase multienzyme complex was resolved into the E1 component and the E2–E3 sub-complex by gel filtration on Sepharose 6B in ethanolamine-phosphate buffer, pH 9.95, at 4°C, as described previously [10]. The proteins were stored at 4°C in 20 mM potassium phosphate buffer, pH 7.0, containing EDTA (1 mg/ml) and sodium azide (0.02% w/v).

Reconstitution of the complex was achieved by mixing gradually increasing amounts of the E1 component with a fixed amount (about 1.3 mg) of E2–E3 sub-complex in 20 mM potassium phosphate/EDTA/sodium azide buffer, pH 7.0, as above, and

leaving the mixtures at 4°C for at least 18 h. The assembled complexes were then separated from any free E1 component by gel filtration on Sepharose 6B in the same buffer, pH 7.0. In other experiments, the self-assembly and the separation of the assembled complexes from free E1 component were carried out in 20 mM potassium phosphate/EDTA/sodium azide buffers of pH 6.0 and 8.0 respectively, also at 4°C.

2.3. *SDS–polyacrylamide gel electrophoresis*

SDS–gels (5%) were run in glass tubes and stained as described previously [11]. Loadings were approx. 10–20 μg protein/component.

2.4. *Measurement of polypeptide chain stoicheiometry*

Samples of complex were treated with methyl [1-^{14}C]acetimidate in 5 M guanidine hydrochloride, the chains were separated by SDS–gel electrophoresis, and the chain ratios determined from the measured radioactivity in the protein bands corresponding with E1, E2 and E3 as described elsewhere [5]. Stained SDS–gels of unamidinated complexes were also examined in a Joyce-Loebl Chromoscan microdensitometer.

2.5. *Ultracentrifugation*

Protein samples (approx. 5 mg/ml) were centrifuged in a Beckman Model E ultracentrifuge at 20°C and the sedimentation followed by Schlieren optics. The runs were carrried out at 25980 rev./min for the enzyme complexes and at 59780 rev./min for the component.

3. Results and discussion

3.1. *Self-assembly at pH 7.0*

The results of a reconstitution experiment carried out at pH 7.0 are shown in figs. 1 and 2. Before analysing the polypeptide chain stoicheiometry by the amidination method [5], the complexes were freed from any unbound E1 component by gel-filtration on Sepharose 6B. The separation by SDS–gel electrophoresis of the three constituent polypeptide chains in the enzyme complexes after amidination is shown in fig.1, the gel samples corresponding to the first 8 points represented in fig.2. It will be seen quite clearly that the binding curve is in fact a straight line for much of its course, that it turns over quite sharply

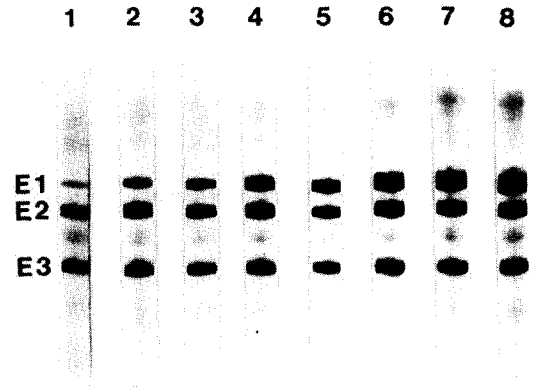

Fig.1. SDS–Gel electrophoresis, after amidination with methyl [1-^{14}C]acetimidate [5], of the first eight enzyme complexes whose polypeptide chain stoicheiometries are given in fig.2.

and that the plateau value for the ratio of E1 : E2 is approx. 1.8 : 1. This implies that the binding of E1 to the E2–E3 sub-complex is extremely tight, which was borne out by the fact that no free E1 could be detected before sample 7 of figs.1 and 2 when the assembly mixtures were gel filtered on Sepharose 6B.

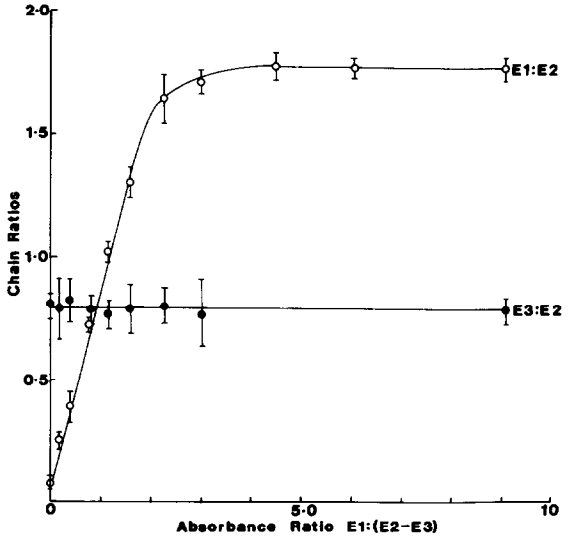

Fig.2. Increasing amounts of E1 were added to fixed amount of E2–E3 sub-complex to yield the mixtures indicated from the absorbance at 280 nm of the two stock solutions. The chain ratios in the purified enzyme complexes were measured by the amidination method [5]: at least four SDS–gels were run for each determination.

The other conclusion drawn from this experiment is that the chain ratio of E1 : E2 can rise to its limit without significant displacement of E3. The chain ratio of E3 : E2 remained approx. 0.8 : 1, the value in the starting sub-complex. This conclusion was reinforced by examining the Coomassie-stained SDS–gels in the microdensitometer, when it was found that the ratio of the colour intensities in the E2 and E3 bands was unchanged throughout the self-assembly. This can be seen also by visual inspection of fig.1.

Samples of the partly assembled complexes were also examined in the ultracentrifuge in the same potassium phosphate buffer, pH 7.0. All gave single, apparently symmetrical peaks. The uncorrected sedimentation coefficient rose steadily from approx. 32 S for the E2–E3 sub-complex to approx. 59 S for the fully assembled complex. These values are in good agreement with those of Reed and co-workers [12], given the differences in experimental conditions. There was no evidence of co-operativity in the binding of E1 to the E2–E3 sub-complex. A similar conclusion has been reached by Busch and Henning [13].

3.2. *Self-assembly at pH 6.0 and pH 8.0*

Reconstitution experiments similar to those described above were also carried out in 20 mM potassium phosphate/EDTA/sodium azide buffers at pH 6.0 and pH 8.0. The plateau values for the chain stoicheiometries determined by the amidination method were 2.1 : 1 : 0.7 and 1.7 : 1 : 0.7, respectively. As before, there was no evidence of substantial displacement of E3 by E1.

3.3. *Ultracentrifugal analysis of the E1 component*

The pyruvate decarboxylase component, E1, has been shown to be a dimer of two polypeptide chains, each of molecular weight approx. 100 000 [3,14] but it has been reported [15] that E1, prepared by resolution of the enzyme complex at pH 9.5, consists of two components separable by ultracentrifugation at pH 7.0 ($s_{20,w}$ values of 9.6 S and 5.6 S). It was suggested that these forms were the dimer and monomer respectively.

Samples of E1 prepared in the present experiments were examined in the ultracentrifuge after dialysis for 24 h at 4°C against 0.1 M potassium phosphate/EDTA/sodium azide buffers at pH 6.0, pH 7.0 and at pH 8.0 and against the same buffer solution adjusted to pH values of 9.0 and 10.0 by addition of ethanolamine. At pH 9 and pH 10 single symmetrical peaks with an apparent sedimentation coefficient of approx. 6.0 S were observed. At pH 7.0 and at 8.0, however, an additional more rapidly sedimenting peak ($s_{apparent}$ = 8.8 S) was visible, as described by Dennert and Eaker [15]. This peak was the major component at both pH values and, indeed, at pH 6.0, the more slowly sedimenting peak was hardly visible. The simplest interpretation is dimer formation from monomers as the pH falls. The reversibility of the dissociation was shown by dialysing a sample of E1 at pH 10.0 for 24 h and then returning it to dialyse at pH 7.0 for a further 24 h, all at 4°C. The major peak (8.8 S) was found to return and to assume approximately the same proportion as in a control sample of E1 held at pH 7.0.

4. Conclusions

These reconstitution experiments support our earlier suggestion that the limiting stoicheiometry (E1 : E2 : E3) of the polypeptide chains in the native *E. coli* pyruvate dehydrogenase complex is 2 : 1 : 1 [5,6]. No obvious cooperativity in binding E1 was detected, in agreement with the results of Busch and Henning [13]. The E1 : E2 ratio reached a maximum value of 2 : 1 in the assembly at pH 6.0 but rose only to about 1.7 : 1 at pH 8.0. The E1 component we used at pH 8.0 was mostly in the dimeric form but some apparent monomers were present. However, at pH 6.0, only a trace of monomeric E1 could be detected. A possible explanation of our results would therefore be that the monomer and the dimer of E1 bound competitively to the same sites on E2 and that the maximum E1 : E2 ratio was only realized at pH 6.0 where E1 was entirely dimeric. Lower E1 : E2 ratios might be expected at higher pH values since E1 begins to dissociate from the enzyme complex at pH 9.0 [12]. Whether the putative monomeric form of E1 described by Dennert and Eaker [15] and by us represents a partly denatured enzyme remains to be seen but the reversibility of its association to dimers suggests that it cannot be badly damaged.

It has already been claimed that the E1 monomer can bind to the E2–E3 sub-complex but that it does not contribute to the catalytic activity [16]. Since the monomeric and dimeric forms of E1 can be separated

by ultracentrifugation (ref. [15] and this work) their interconversion must be slow. A rigorous analysis of the kinetics of the interconversion and of the reconstitution of the complex from the two forms of E1 is now called for.

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References

[1] Reed, L. J. and Oliver, R. M. (1968) Brookhaven Symposia in Biology 21, 397–412.
[2] Reed, L. J. (1974) Accounts of Chemical Research 7, 40–46.
[3] Eley, M. H., Namihara, G., Hamilton, L., Munk, P. and Reed, L. J. (1972) Arch. Biochem. Biophys. 152, 655–669.
[4] Vogel, O., Hoehn, B. and Henning, U. (1972) Proc. Natl. Acad. Sci. USA 69, 1615–1619.
[5] Bates, D. L., Harrison, R. A. and Perham, R. N. (1975) FEBS Lett. 60, 427–430.
[6] Perham, R. N. (1975) Phil. Trans. Roy. Soc. Lond. B. 272, 123–136.
[7] Reed, L. J., Pettit, F. H., Eley, M. H., Hamilton, L., Collins, J. H. and Oliver, R. M. (1975) Proc. Natl. Acad. Sci. USA 72, 3068–3072.
[8] Reed, L. J. and Mukherjee, B. B. (1969) in: Methods in Enzymology (Colowick, S. P. and Kaplan, N. O. eds) 13, 55–61, Academic Press, London.
[9] Bates, D. L., Coggins, J. R. and Perham, R. N. (1975) Analyt. Biochem. 68, 175–184.
[10] Coggins, J. R., Hooper, E. A. and Perham, R. N. (1976) Biochemistry 15, 2527–2533.
[11] Perham, R. N. and Thomas, J. O. (1971) FEBS Lett. 15, 8–12.
[12] Koike, M., Reed, L. J. and Carroll, W. R. (1963) J. Biol. Chem. 238, 30–39.
[13] Busch, W. and Henning, U. (1972) unpublished work quoted by Henning, U., Vogel, O., Busch, W. and Flatgaard, J. E. (1972) in: Protein–Protein Interactions (Jaenicke, R. and Helmreich, E. eds) pp. 343–361, Springer-Verlag Berlin.
[14] Vogel, O. and Henning, U. (1971) Eur. J. Biochem. 18, 103–115.
[15] Dennert, G. and Eaker, D. (1970) FEBS Lett. 6, 257–261.
[16] Dennert, G. unpublished work quoted in ref. [15].