THE STOICHIOMETRY OF POLYPEPTIDE CHAINS IN THE PYRUVATE DEHYDROGENASE MULTIENZYME COMPLEX OF *E. COLI* DETERMINED BY A SIMPLE NOVEL METHOD

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1. Introduction

Many enzymes and other proteins possess quaternary structure [1]. In simple cases, the sub-units are identical and a knowledge of the mol. wt. of the native enzyme and of its denatured sub-units suffices to establish the number of protein chains in the oligomer. With larger structures, however, evidence may often have to be drawn from other sources, such as X-ray diffraction analysis, electron microscopy and arguments from symmetry [1,2].

Multienzyme complexes [3] can present unusually awkward problems since they contain more than one type of polypeptide chain, the stoichiometry of which has to be determined. The pyruvate dehydrogenase multienzyme complex of *E. coli* contains three different types of polypeptide chain, responsible for the three component enzyme activities: these are E1, pyruvate decarboxylase; E2, lipoyl transacetylase; and E3, lipoamide dehydrogenase (for reviews, see [4,5]). There is, however, no agreement on the chain ratio of E1 : E2 : E3 in the native complex. A ratio of 2 : 2 : 1 is favoured by Reed and his colleagues [5,6] but it has been reported [7] that some preparations of the enzyme carry a molar excess of E1 compared with E2 and E3 (about 1.3 : 1 : 1). This 'excess' E1 can be stripped off by chromatography of the enzyme complex on columns of calcium phosphate gel, the chain ratio for the resulting 'core' complex then being 1 : 1 : 1 [7].

In the present paper, we describe a new method of determining stoichiometry that is reliable and rapid: the method is equally applicable to mixtures of non-complexed proteins.

2. Materials and methods

2.1. *Enzymes and reagents*

Fructose diphosphate aldolase (EC 4.1.2.13) and glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) from rabbit muscle were purchased from Boehringer, tryptophan synthase (EC 4.2.1.20) from *E. coli* was a gift from Dr T. Creighton and the pyruvate dehydrogenase multienzyme complex was prepared from *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 approx. 450 000 dpm/μmol. Guanidine hydrochloride was recrystallized twice from 96% ethanol. All other chemicals were of A.R. grade and were used without further purification.

2.2. *Amidination of proteins*

Protein samples of up to 1 mg were dialysed against 1 mM EDTA, pH 7.0, and freeze-dried. The samples were dissolved in 0.1 ml of 0.2 M sodium borate buffer, pH 8.5, containing 5 M guanidine hydrochloride, 2 mM EDTA and 0.01% (w/v) sodium azide. A solution of 1 M methyl[1-^{14}C]acetimidate was prepared from the hydrochloride [9] and 0.01 ml of this solution was added to the protein samples (final reagent concentration of 0.1 M). Reaction was allowed to continue for at least 5 h at room temperature (20°C). The samples were then diluted to 0.5 ml with 20 mM sodium phosphate buffer, pH 7.0, and dialysed against 20 mM sodium phosphate buffer, pH 7.0, containing 2 mM EDTA, 0.1% (w/v) SDS and 0.01% (w/v) sodium azide to remove excess radiolabel.

2.3. *SDS-polyacrylamide gel electrophoresis*

SDS-gels (5% and 7.5%) were run in glass tubes and stained as described previously [10]. The gels were 7.0 × 0.5 cm and loadings were approx. 10–20 μg protein per component.

2.4. *Determination of radioactivity*

After staining, the protein bands were excised and the pieces of gel dried under vacuum in plastic scintillation vials. The dried gels were dissolved by incubation for about 6 h at 60°C with 0.3 ml of 100 vol hydrogen peroxide. Control experiments showed that there was no loss of radiolabel caused by this treatment. Scintillant (3.0 ml of toluene–triton (2 : 1, by vol) containing 2,5-diphenyloxazole (5 g/l)) was added and the clear samples were counted in a Nuclear Chicago Unilux II scintillation counter. The ^{133}Ba external standard calibrated against a series of sealed, quenched samples (Amersham-Searle) was used to correct for quenching. The efficiency was generally 70–75% and blank gel slices put through the entire procedure were counted for background radioactivity.

2.5. *Protein concentration*

Approximate protein concentrations were determined spectrophotometrically, assuming $E^{1\%}_{280} = 10$. Accurate protein concentrations were determined by amino acid analysis with a Beckman 120C analyser as described previously [11].

2.6. *Calculation of stoichiometry of polypeptide chains*

D_i, the radioactivity (in dpm) in component i of a series of proteins resolved by gel electrophoresis is given by

$$D_i = S \cdot E_i \cdot L_i \cdot X_i$$

where S = specific radioactivity of the reagent
E_i = extent of the modification reaction
L_i = number of amino groups/mol of component i
X_i = number of moles of component i

For any two components i and j, provided that $E_i = E_j$

$$\frac{X_i}{X_j} = \frac{D_i}{L_i} \div \frac{D_j}{L_j}$$

The specific radioactivity of the reagent therefore need not be determined.

3. Results

3.1. *Tests of the method*

The method was tested first with an artificial mixture of two proteins. Mixtures of aldolase and glyceraldehyde 3-phosphate dehydrogenase ranging from 4 : 1 to 1 : 4 (v/v) were prepared from stock solutions of the pure enzymes in 20 mM sodium phosphate buffer, pH 7.0. The relative molarity of the stock solutions was 0.78 : 1 (aldolase : glyceraldehyde 3-phosphate dehydrogenase). Samples of the mixtures were then analysed by the amidination procedure described above.

The results are shown in fig.1. The lysine content of aldolase was taken as 26.6 residues/subunit (mol. wt. 40 000) [11] and that of glyceraldehyde 3-phosphate dehydrogenase as 27.0 residues/subunit (mol. wt. 36 000) [12]. The line has the theoretical slope of 1.0 and the points are the experimentally determined ratios. A least squares analysis gives a gradient of 1.03 for the experimental points.

Tryptophan synthase from *E. coli* is a tetramer of the type $\alpha_2\beta_2$ [13]. The experimentally determined ratio of ^{14}C in the amidinated chains (α : β) was 0.70 ± 0.01. The lysine content of the α-chain is reported to be 13.0 residues/subunit (mol. wt. 39 000

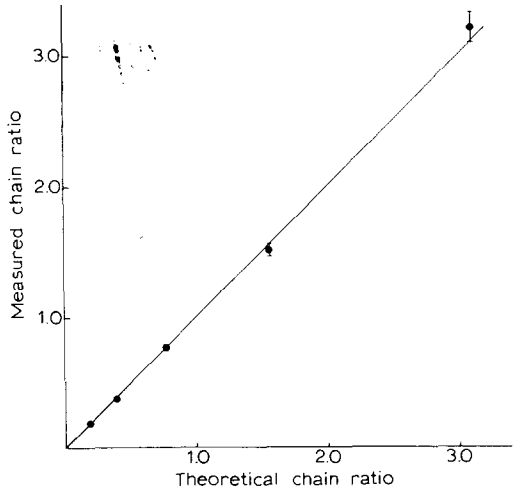

Fig.1. Comparison of the theoretical and measured ratios of the polypeptide chains of aldolase: glyceraldehyde 3-phosphate dehydrogenase in an artificial mixture.

[13] and of the β-chain 18.3 residues/subunit (mol. wt. 44 200) [13,14]. Our results therefore indicate a chain ratio (α : β) of 0.99 ± 0.02.

3.2. *The pyruvate dehydrogenase multienzyme complex*

The pattern of three protein bands on SDS-gel electrophoresis of amidinated complex is identical with that of unmodified complex [10]. The results of an analysis of five different samples of the multienzyme complex from *E. coli* are given in table 1. Two samples (1 and 2) were purified from *E. coli* (Crookes strain) and the other three (3,4 and 5) from a pyruvate dehydrogenase complex-constitutive mutant of *E. coli* K12. The results have been calculated in three ways. The first uses the amino acid analyses of the component chains determined in this laboratory [15], the other two use those obtained elsewhere [6, 16, 17]. No differences in amino acid compositions of components from different strains have been detected in this laboratory. The results of the three sets of calculations are in reasonable agreement. We have chosen E2 as unity in calculating stoichiometries since this component is known to be the structural 'core' around which E1 and E3 are assembled [4,5].

In all cases there is a molar excess of E1 chains compared with E2 chains whereas the chain ratio for E3/E2, although variable, never significantly exceeds unity. These results therefore agree quite well with those of Vogel et al. [7] except that we find the ratio of E1/E2 approaches a value of 2 on occasion (samples 3 and 4).

4. Discussion

The amidination method for determining chain ratios described above offers advantages of speed and simplicity compared with conventional gel scanning procedures which, to be reliable, demand careful, even laborious, calibration [7]. Since the proteins in a

Table 1
Stoichiometry of polypeptide chains in the pyruvate dehydrogenase multienzyme complex of *E. coli*

Sample	Number of gels run	Ratio of ^{14}C dpm E1 : E2 : E3 (standard deviation in parentheses)	Stoicheiometry E1 : E2 : E3
1 (Crookes strain)	3	1.20 (0.04) : 1 : 0.79 (0.02)	1.38 : 1 : 1.09[a] 1.29 : 1 : 0.94[b] 1.64 : 1 : 1.25[c]
2 (Crookes strain)	4	1.18 (0.08) : 1 : 0.55 (0.02)	1.36 : 1 : 0.76[a] 1.27 : 1 : 0.65[b] 1.61 : 1 : 0.88[c]
3 (constitutive strain of K12)	12	1.46 (0.07) : 1 : 0.63 (0.05)	1.67 : 1 : 0.87[a] 1.57 : 1 : 0.75[b] 1.99 : 1 : 1.01[c]
4 (constitutive strain of K12)	8	1.58 (0.09) : 1 : 0.64 (0.04)	1.81 : 1 : 0.88[a] 1.69 : 1 : 0.76[b] 2.15 : 1 : 1.02[c]
5 (constitutive strain of K12)	2	1.20 : 1 : 0.62	1.38 : 1 : 0.85[a] 1.29 : 1 : 0.73[b] 1.64 : 1 : 0.98[c]

[a] mol lysine/subunit mol. wt. [15] : E1, 52.5/100 000; E2, 60.1/80 000; E3, 43.5/55 000.
[b] mol lysine/subunit mol. wt. [6] : E1, 47.9/100 000; E2, 51.4/70 000; E3, as ([a]) above.
[c] mol lysine/subunit mol. wt. [16,17,20] : E1, 47.7/100 000; E2, 65.0/80 000; E3, 41.0/56 000.

sample are amidinated under strongly denaturing conditions in the same vessel, it is likely that all components will be modified to the same extent. The conditions we have chosen give greater than 90% modification of the amino groups in proteins tested in this laboratory. Amidination is specific for amino groups and causes no change of charge at the modified lysine residue [18]. Modified proteins therefore migrate on SDS-gel electrophoresis in the same place as the unmodified proteins and are easily identified. And, finally, since most proteins contain a large number of lysine residues, the incorporation of radiolabel is greatly multiplied and the contribution from possible reaction at the N-terminus can be safely neglected. All the pre-requisites for a good radiolabelling method are therefore met and the tests with a synthetic mixture of proteins and with tryptophan synthase demonstrate its reliability.

The samples of the pyruvate dehydrogenase multienzyme complex all carry a molar excess of E1 chains and the ratio of E1 : E2 can approach 2 (samples 3 and 4) (table 1). Sample 5 came from the same culture of *E. coli* K12 as sample 4 but precipitated at a different pH in the iso-electric precipitation of the enzyme during purification [8]. Whether the variable stoichiometry is due to partial loss of subunits during enzyme purification or to the existence of partly assembled complexes in vivo remains to be determined. What is clear is that, unlike the smaller oligomeric enzymes, enzymically active pyruvate dehydrogenase multienzyme complexes of variable subunit composition are permitted [1,19].

The pyruvate dehydrogenase complex of *E. coli* has a transacetylase core, probably of octahedral (432) symmetry, which demands 24 packing units, probably polypeptide chains, for its assembly [4,5,10]. If we accept the chain ratio of E1 : E2 : E3 of 2 : 1 : 1 as representing the upper limit of the stoichiometry, strict octahedral symmetry for the assembled complex can only be preserved if the packing unit for E1 is the dimer i.e. the bound dimer is asymmetric. The effect of variable subunit composition on the enzymic activity of the complex now needs to be investigated more closely.

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