SUBUNIT EXCHANGE IN THE PYRUVATE DEHYDROGENASE COMPLEX OF *ESCHERICHIA COLI*

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1. Introduction

The pyruvate dehydrogenase complex of *E. coli* [1,2] contains multiple copies of 3 proteins responsible for the constituent enzyme activities: pyruvate decarboxylase (E1); lipoate acetyltransferase (E2); lipoamide dehydrogenase (E3). The E2 component forms a structural core to which the E1 and E3 components bind independently and appears to comprise 24 polypeptide chains arranged with octahedral symmetry [3,4]. Each E2 chain contains two residues of lipoic acid which are reductively acetylated during the course of the normal enzyme reaction [5–7]. We have shown that the lipoyl moieties are connected by a remarkable system of active-site coupling [8,9], a result confirmed and extended in [7,10,11]. The simplest explanation was that the initial reductive acetylation of lipoic acid residues could be followed by extensive intramolecular transacetylation reactions between lipoyl groups on neighbouring E2 chains of the core [7,8]. Pulsed quenched-flow experiments demonstrated that these postulated transacetylation reactions are fast and not rate determining in the reaction mechanism of the complex [12].

Our study of the self assembly of the enzyme complex in vitro [8] also demonstrated that E1 dimers bind very tightly to the E2E3 subcomplex, with a K_a probably $> 10^7$ M^{-1}. In this paper, we describe measurements of the rate constant for dissociation of the E1 component from the intact complex. As expected, this rate is much too slow to account for active-site coupling by an alternative mechanism of continual dissociation and reassociation of the E1 and E2E3 components.

2. Materials and methods

Pyruvate dehydrogenase complex was purified from *E. coli* K12 [4] and was resolved into the E2E3 subcomplex and the free E1 component by gel filtration at pH 10 [13]. The overall complex activity was assayed by NAD^+ reduction in the presence of pyruvate [9]. Protein concentrations were calculated from A_{280} [14].

Intrinsically ^{35}S-labelled complex was isolated from *E. coli* grown in the presence of [^{35}S]sulphate [15]. It was diluted 10-fold with unlabelled complex to a final specific radioactivity of $\sim 10^6$ dpm/mg. The polypeptide chain stoicheiometry of the ^{35}S-labelled complex was calculated from measurements of the radioactivity in each of the 3 protein bands separated by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis [15].

The complex was inhibited by incubation with 2 mM *N*-ethylmaleimide at 0°C in the presence of 1 mM pyruvate, 0.5 mM thiamin pyrophosphate, 5 mM $MgCl_2$ and 0.4 mM NAD^+ [5]. After 20 min, the reaction was terminated by the addition of 2-mercaptoethanol. The enzyme was dialysed against 50 mM sodium phosphate buffer (pH 7.0) containing EDTA (1 g/l) and sodium azide (0.1 g/l). The catalytic activity was <5% of that of native complex.

A mixture of ^{35}S-labelled complex and free unlabelled E1 was prepared in 20 mM sodium phosphate buffer, (pH 7.0) containing EDTA (1 g/l) and sodium azide (0.2 g/l) and at various times the 2 species were separated by gel filtration at 4°C on a column of Sepharose 6B (550 × 6.5 mm) in the same buffer. The flow rate was 5.4 ml/h. Fractions were

monitored for radioactivity by scintillation counting [15] and for protein content by their A_{280}.

3. Results

3.1. *Migration of E1 subunits from* N-*ethylmaleimide-inhibited complex to active E2E3 subcomplex*

In the presence of pyruvate the complex can be specifically inhibited with *N*-ethylmaleimide, which reacts with the reductively acetylated lipoic acid residues, leaving the E1 component apparently unaffected [5,16]. Consequently, if inhibited complex and E2E3 subcomplex are mixed, any E1 subunits dissociating from the former can bind to the latter, which should be accompanied by the appearance of overall enzyme activity [8].

Stock solutions of E2E3 subcomplex (3 mg/ml) and *N*-ethylmaleimide-inhibited complex (5 mg/ml) were prepared as described above. The molarity of E2, and hence of E1 binding sites, in the two solutions was approximately equal. The residual pyruvate dehydrogenase specific activity in each solution was <5% of that of native complex. Therefore, if the E1 were to redistribute itself randomly between subcomplex and inactivated complex, the enzyme activity of the mixture should rise to ~25% of that of the native complex. Equal volumes of the two solutions were mixed and incubated at 0°C with samples being taken for enzyme assay at intervals. There was no discernible increase in catalytic activity even after 24 h incubation. The integrity of the subcomplex was then checked by adding pure E1 to an assay cuvette containing the above mixture. A rapid appear ance of activity ensued.

3.2. *Exchange of E1 subunits between* ^{35}S-*radiolabelled complex and unlabelled free E1*

Pyruvate dehydrogenase complex intrinsically radiolabelled with ^{35}S was incubated with an excess of unlabelled free E1 and, after various times, the two species were separated by gel filtration on Sepharose 6B, as described above. The rate of E1 exchange was monitored by measuring the decrease in radioactivity of the E1 bound to the complex and the corresponding increase in radioactivity in the free pool of E1 subunits.

Preliminary experiments established that no detectable exchange took place at 0°C over a period of several hours, in accord with result obtained with *N*-ethylmaleimide-inhibited complex. More detailed work was therefore carried out at a higher temperature (15°C). A mixture of ^{35}S-labelled complex (2.5 mg/ml) with polypeptide chain ratios (E1:E2:E3) of 1.59 : 1 : 0.89 and unlabelled E1 (10.5 mg/ml) was incubated at 15°C for 8 days and at various times a sample (0.14 ml) was removed and gel filtered on a column of Sepharose 6B. From the elution profiles it was apparent that radioactivity was slowly being displaced from the higher to the lower molecular weight species.

The specific enzyme activity of the gel filtered complex remained constant throughout the experiment (25 μmol $NAD^+.min^{-1}.mg\ complex^{-1}$). The specific radioactivity of the E1 separated by gel filtration was calculated from the measurements of radioactivity and A_{280} of the column effluent. Samples of the enzyme complex after gel filtration were analysed by SDS–polyacrylamide gel electrophoresis and the polypeptide chain ratios were determined by measuring the radioactivity in each protein band. No proteolytic degradation of the component enzymes was observed during the course of the experiment [17,18]. As a control, a sample of the stock solution of ^{35}S-radiolabelled complex was gel filtered under identical conditions. This was used to establish the zero time point in the subsequent analysis. The results of this experiment are presented in fig.1. A slow exchange of E1 subunits was observed and, as expected, the disappearance of bound ^{35}S-labelled E1 was matched by the appearance of radioactivity in the pool of free E1.

The interactions between the E1 and E2 components of the pyruvate dehydrogenase complex are non-covalent. We may therefore write:

$$E1E2E3 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E1 + E2E3$$

where k_1 is the rate constant for the dissociation of E1 from the complex, and k_{-1}, the rate constant for the assembly process. (Strictly, these apply to the first E1 subunit to dissociate or the last to bind.) Assembly is known to be very rapid as it is possible to obtain active complex within seconds of mixing catalytic amounts of the free E1 component with E2E3 subcomplex in the standard assay system (e.g.,

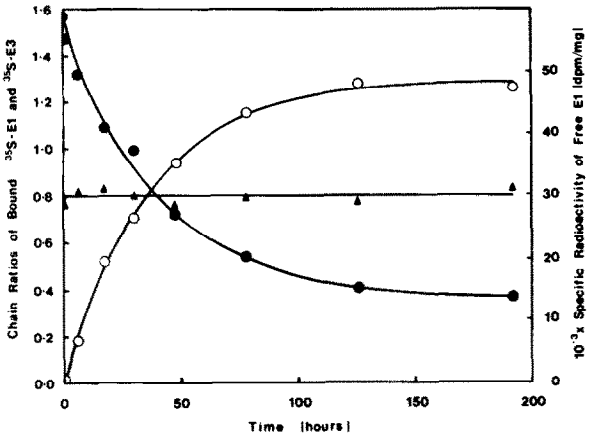

Fig.1. Exchange of ^{35}S-labelled E1, bound to the pyruvate dehydrogenase complex of *E. coli*, with free unlabelled E1. Samples were taken at various times from a mixture of ^{35}S-labelled complex (2.5 mg/ml) and unlabelled E1 (10.5 mg/ml) and the two species were separated by gel filtration. The complex was analysed by SDS–gel electrophoresis. The [^{35}S]E1 : [^{35}S]E2 polypeptide chain ratio (●) and the [^{35}S]E3 : [^{35}S]E2 polypeptide chain ratio (▲) were measured. The specific radioactivity of the free E1 component was also determined (○).

[19]). Since the concentration of enzyme binding sites in a typical assay is $\sim 10^{-8}$ M, this implies that the second order rate constant for the association reaction must be $>10^7$ $M^{-1}.s^{-1}$. Therefore, in our experiment, the concentration of free E2E3 subcomplex was negligibly small. However, there was initially a large concentration of free unlabelled E1 subunits and so the amount of radioactive E1 bound to the complex decreased with time as a function of the rate constant k_1.

A non-linear least squares analysis of the data in fig.1 gave the following values for k_1:

(a) From loss of complex-bound [^{35}S]E1
$7.1\ (\pm 0.8) \times 10^{-6}\ s^{-1}$;

(b) From increase in free [^{35}S]E1
$7.6\ (\pm 0.3) \times 10^{-6}\ s^{-1}$.

These values correspond to a half-time of ~27 h. They are not corrected for the increasing radioactivity in the pool of free E1, which would cause the observed rate constant to be greater than the true rate constant by ~10% under our conditions. The limiting polypeptide chain ratio of [^{35}S]E1 : [^{35}S]E2 in the complex was found to be 0.37 (± 0.04) whereas the expected value calculated from the ratio of ^{35}S-labelled and unlabelled E1 in the starting mixture was 0.17. This means that a small fraction (almost 13%) of the ^{35}S-labelled E1 was bound to the E2 core more strongly than the rest. This may be due to unidentifiable changes in the enzyme complex or E1 subunits during the prolonged incubation of the exchange experiment and ought not to be over-interpreted at this stage.

The polypeptide chain ratio of [^{35}S]E3 : [^{35}S]E2 fell from a value of 0.89 (± 0.02) in the starting complex to an average of 0.80 (± 0.03) in the gel-filtered complexes, as shown in fig.1. This small decrease was not due to displacement of E3 subunits by E1 [20] because it occurred equally in all samples, even those to which no free E1 had been added. Any contribution these radioactive E3 molecules made to the measured specific radioactivity of free E1 after gel filtration was allowed for by the control experiment.

4. Discussion

Dissociation of the E1 component from the pyruvate dehydrogenase complex of *E. coli* is a very slow process at pH 7.0. Having measured the rate constant for dissociation ($\sim 7 \times 10^{-6}\ s^{-1}$ at 15°C), assuming a lower limit of the rate constant for association (10^7 $M^{-1}.s^{-1}$ at 30°C) and making an approximate allowance for the temperature difference, we estimate the binding constant to be $>10^{11}$ M^{-1}. More sophisticated experiments would be required to obtain a more accurate value for the rate constant for association and hence for the binding constant. Our experiments appear to exclude completely any possibility that a large number of lipoic acid residues on the E2 core can be directly acetylated by a single E1 dimer which migrates around the complex by a process of dissociation and reassociation. Direct transacetylation reactions among lipoic acid residues remain the most likely explanation of active-site coupling.

Mammalian pyruvate dehydrogenase complexes also possess the property of multiple servicing of lipoic acid residues by a single E1 molecule ([21], C. J. Stanley, L. C. Packman, C. E. Henderson, M. J. Danson and R. N. Perham, unpublished work). However, using techniques different from the ones we

describe above, Cate and Roche [21] have demonstrated that exchange of E1 subunits in a mammalian complex can occur within a few minutes at 20°C and conclude that it makes a significant contribution to the extent of acetylation, although the rate is small compared with the turnover number of the enzyme. On the other hand, these authors suggest that this process may be important in the regulation of the complex activity by the enzyme-bound kinase that phosphorylates E1.

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