Restoration of β -Galactosidase to *Escherichia coli* M15

COMPLEMENTATION STUDIES

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Carboxymethylated β -galactosidase from *Escherichia coli* was dissociated at 100°C to form carboxymethylated fragments A and B. The mol.wts. of carboxymethylated fragments A and B were determined by gel filtration to be 64300 and 22400 respectively. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of carboxymethylated fragments A and B that had been pretreated with 2-mercaptoethanol and sodium dodecyl sulphate yielded mol.wts. of 64000 and 22100 respectively. Carboxymethylated fragments A and B had arginine as their C-terminal amino acid. When a crude extract of *E. coli* M15 was filtered through a column of Sepharose 6B, it was found that carboxymethylated fragment B could restore β -galactosidase activity when added to fractions having mol.wts. estimated to be 123000, 262000 and 506000. These fractions are referred to as 'complementable fractions'. Similarly, it was found that carboxymethylated fragment A could restore enzyme activity to fractions having mol.wts. estimated to be 63000, 253000 and 506000. Estimates of the molecular weights of the β -galactosidase activity obtained by restoration with carboxymethylated fragments A and B were made by filtering the active enzyme through another column of Sepharose 6B. The enzyme obtained by complementation with carboxymethylated fragment B, i.e. the complemented enzyme, had mol.wt. 525000, and that obtained with carboxymethylated fragment A had mol.wts. of 525000, 646000 and 2000000. The latter finding suggests that multiple forms of complemented β -galactosidase can exist.

Complementation *in vitro* is the restoration of enzyme activity by mixing two enzymically inactive protein fractions. *Escherichia coli* M15 lacks active β -galactosidase. The structural locus for this enzyme, the *z* gene, can be divided into three segments, α , β and ω . Mutant M15 has a deletion in the α segment (Ullmann *et al.*, 1968). It is possible to correct the enzyme defect of the mutant by adding the product of the deleted region, α -donor, i.e. by complementation. Complementation studies with extracts of mutant strains of *E. coli* indicated the presence of different subunits in β -galactosidase (Ullmann *et al.*, 1965, 1967). Apparently the subunits of β -galactosidase from wild-type and mutant M15 are both constructed from smaller fragments. We have demonstrated that the subunits and fragments of β -galactosidase obtained by enzyme dissociation have mol.wts. in the range 130000–23500. Complementation studies showed that dissociation products having mol.wts. between 100000 and 23500 complemented an unfractionated crude extract of mutant M15. The fragment of mol.wt. 23500 exhibited high α -donor activity with mutant M15 (Marinkovic *et al.*, 1975).

It has been shown that five different α -acceptors are present in mutant M15 (Marinkovic & Tang, 1971). With a CNBr fragment of β -galactosidase from *E. coli* as the α -donor, we obtained two complementable fractions and one complemented enzyme from mutant M15 (Marinkovic & Marinkovic, 1976). Goldberg (1969) purified an ω -complemented enzyme from the diploid strain MU 366/B9. The B9 mutant is the largest deletion in the α and β region but it still has ω -activity. The ω -acceptor MU 366 is an amber mutant, and the chain-terminating codon occurs in the ω -region. This enzyme is a tetramer with mol.wt. 595000. Ullmann *et al.* (1968) showed that a complemented enzyme from strain S908/B9 has a sedimentation coefficient of 15.8S, which is identical with that of wild-type β -galactosidase obtained by sucrose-density-gradient centrifugation. The ω -acceptor S908 is also an amber mutant. In the present investigation we dissociated carboxymethylated wild-type β -galactosidase into two carboxymethylated fragments, A and B. By using these fragments as α -donor, we determined the number of complementable fractions and comple-

mented enzymes that could be obtained when crude extract of mutant M15 was separated on a column of Sepharose 6B.

Experimental

Materials

Sephadex G-100 and G-25, Sepharose 6B and Blue Dextran 2000 were obtained from Pharmacia, Uppsala, Sweden. *o*-Nitrophenyl β -D-galactoside was from Mann Research Laboratories, New York, NY, U.S.A. *E. coli* K12 strain 3300 was obtained from Dr. E. Steers, Jr., National Institutes of Health, Bethesda, MD, U.S.A. β -Galactosidase from *E. coli* K12 strain 3300 was purified as described previously (Marinkovic & Marinkovic, 1976). *E. coli* E 706/M15 was obtained from Dr. J. R. Beckwith, Harvard Medical School, Boston, MA, U.S.A., and was grown as described previously (Marinkovic & Marinkovic, 1976). Iodo[^3H]acetic acid (100–300 mCi/mmol) was obtained from New England Nuclear Corp., Boston, MA, U.S.A. All other chemicals used were A.R. grade.

Methods

Carboxymethylation. β -Galactosidase was carboxymethylated as described by Craven *et al.* (1965), with slight modification. First, 100 mg of β -galactosidase was dissolved in 20 ml of 0.1 M- NH_4HCO_3 (containing 0.01 M-EDTA) and 6 M-guanidinium chloride. Dithiothreitol (0.1 M) was added, and then a stream of N_2 was gently passed over the surface of the reaction mixture for approx. 5 min. The sample was sealed and then heated in a water bath at 100°C for 2 h, after which time iodoacetate was introduced in a tenfold molar excess over the dithiothreitol. The final product was dialysed against 0.01 M- NH_4HCO_3 at 20°C for 2 days, and then freeze-dried.

Heating the carboxymethylated β -galactosidase. Carboxymethylated β -galactosidase (20 mg) was dissolved in 0.04 M-Tris/citric acid buffer, pH 7.5, containing 8 M-urea. The tube was sealed and then heated in a water bath at 100°C for 5 h. The sample was dialysed overnight at room temperature (20°C) against 0.1 M-ammonium acetate buffer, pH 8.5, containing 0.1% sodium dodecyl sulphate. It was then applied to a column of Sephadex G-100.

Sephadex gel filtration. The carboxymethylated fragments A and B were separated on a column (150 cm $\times$ 2 cm diam.) of Sephadex G-100 equilibrated in 0.1 M-ammonium acetate buffer, pH 8.5, containing 0.1% sodium dodecyl sulphate. Carboxymethylated fragments were dissolved in the same solution and applied to the column. Fractions (5 ml) were collected at a flow rate of 15 ml/h. Peaks containing carboxymethylated fragments A and B were pooled and dialysed overnight at 20°C against 25% (v/v) acetic acid. To remove sodium dodecyl sulphate

before C-terminal determinations, carboxymethylated fragments A and B were applied to a column (40 cm $\times$ 2 cm diam.) of Sephadex G-25 which had been equilibrated at 20°C with 25% (v/v) acetic acid.

Molecular-weight determination of carboxymethylated fragments A and B. The molecular sizes of carboxymethylated fragments A and B were investigated with a column (150 cm $\times$ 2 cm diam.) of Sephadex G-100 equilibrated and eluted in 0.1 M-ammonium acetate buffer, pH 8.5, containing 0.1% sodium dodecyl sulphate. The column was calibrated with the following standards (mol.wts. in parentheses): bovine albumin (66 500), from Sigma, St. Louis, MO, U.S.A.; ovalbumin (45 000), from Pentex, Kankakee, IL, U.S.A.; α -chymotrypsin (22 500); ribonuclease (12 500), both from Sigma. A plot of the elution volumes of the standards against the logarithms of their molecular weights was used to estimate the molecular weight of the carboxymethylated fragments.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Fragments A and B were carboxy[^3H]-methylated as described previously (Marinkovic & Marinkovic, 1977). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the fragments was carried out as described by Fairbanks *et al.* (1971). The acrylamide concentration was 5.6% (w/v). The gels were stained with Coomassie Brilliant Blue R-250. The gel columns were sliced into 2 mm-thick sections which were incubated overnight at 50°C with 0.5 ml of 30% (v/v) H_2O_2 . Then 10 ml of scintillation fluid (from Amersham/Searle, IL, U.S.A.) was added to each section and ^3H radioactivity counted in a Beckman LS-150 liquid-scintillation system.

C-Terminal amino acid analyses. Carboxymethylated fragments A and B (1.5 mg/0.1 ml) were hydrolysed with di-isopropyl phosphorofluoridate-treated carboxypeptidase B1 (purified by Marinkovic *et al.*, 1977) under conditions similar to those described by Ambler (1972). The fragments were dissolved in 0.2 M-N-ethylmorpholine adjusted to pH 8.5 (with acetic acid), and then incubated for 4 h at 37°C. The reaction was stopped by the addition of an equal volume of acetic acid, the sample was centrifuged in a Sorvall centrifuge for 20 min at 20°C at 10 000 g/min, and the clear supernatant freeze-dried and analysed for liberated amino acids with a Beckman amino acid analyser.

Sepharose gel filtration. A Sepharose 6B column (122 cm $\times$ 3 cm diam.) was equilibrated and eluted with the same solvent at 20°C with 0.05 M- $\text{NaH}_2\text{PO}_4/\text{NaOH}$ buffer, pH 7.0, containing 1 mM- MgCl_2 , 2 mM-EDTA and 0.01 M-2-mercaptoethanol. A crude extract of mutant M15 (about 300 or 900 mg of protein) was obtained as described previously (Marinkovic & Marinkovic, 1976) and applied to the column. Fractions (about 5 ml) were collected at a flow rate of 35 ml/h. The fractions were screened with

carboxymethylated fragments A and B for complementation activity.

Molecular-weight determination of the complementable fractions. The molecular weights of the complementable fractions and complemented enzymes were determined with the above column of Sepharose 6B as described by Whitaker (1963). The column was calibrated with the following standards (mol.wts. in parentheses): β -galactosidase (1080000 and 540000) purified from *E. coli* K12 3300 as described previously (Marinkovic & Marinkovic, 1976); pig thyroglobulin (660000) and bovine albumin (66500), from Sigma; human γ -globulin (fraction III; 157000), from Mann Research Division at Becton, Dickinson, New York, NY, U.S.A.; ovalbumin (45000), from Pentex. A plot of the elution volumes of the standards against the logarithms of their molecular weights was used to estimate the molecular weight of complementable fractions and complemented enzymes.

$(\text{NH}_4)_2\text{SO}_4$ precipitation. The eluates from the Sepharose 6B column were pooled into six complementable fractions, D₁, D₂, D₃, C₁, C₂, C₃. Solid $(\text{NH}_4)_2\text{SO}_4$ was added at 20°C to each fraction to 50% (0.313 g/ml) saturation. The precipitates were collected by centrifugation and then dissolved in 0.05M- NaH_2PO_4 /NaOH buffer, pH 7.0, containing 1mM- MgCl_2 , 2mM-EDTA and 0.01M-2-mercaptoethanol. The solutions were dialysed against this buffer at 4°C for 2 days. Small amounts of insoluble material that formed were removed by centrifugation in a Sorvall centrifuge for 10 min at 4°C at 10000g-min. The fractions were then incubated with carboxymethylated fragments A and B, and estimates of the molecular weights of the complemented enzymes made as described above.

Incubation to form complemented enzymes. The incubation mixtures were composed of 4 ml of 0.1M- NaH_2PO_4 /NaOH buffer, pH 7.0, containing 1mM- MgCl_2 , 0.01M-2-mercaptoethanol and 1mg of carboxymethylated fragment A or B. The following amounts of α -acceptor fractions were used: C₁, 18 mg; C₂, 28 mg; C₃, 84 mg; D₁, 31.5 mg; D₂, 45 mg; D₃, 171 mg. Protein was determined by the micro biuret method of Goa (1953). The carboxymethylated fragments were freeze-dried and the sodium dodecyl sulphate was removed before they were dissolved in 0.02M-Tris/HCl buffer, pH 7.2, containing 0.01M-EDTA, 0.01M-NaCl and 0.1M-2-mercaptoethanol. The mixtures were incubated at 28°C for 48 h.

Complementation assay. Samples (0.1 ml) of the crude extract of mutant M15 [partially purified as described previously (Marinkovic & Marinkovic, 1976) and as described above] from each fraction from the Sepharose 6B column were used as the α -acceptor. The carboxymethylated fragments A and B, dissolved in 0.02M-Tris/HCl buffer, pH 7.2, containing 0.01M-EDTA, 0.01M-NaCl and 0.10M-2-mercaptoethanol, were tested as α -donors in the complementation

assay. For the assay of complementation activity the method described previously (Marinkovic & Marinkovic, 1976) was used. The α -donor activity was expressed as units of β -galactosidase after 16 h incubation at 28°C. The units of complementation activity in the sample were calculated as described by Pardee *et al.* (1959).

Results

Molecular weight of carboxymethylated fragments with α -donor activity

Gel filtration. For the determination of the molecular weights of carboxymethylated fragments A and B, gel filtration was performed with marker proteins run under conditions identical with those used for the carboxymethylated fragments (Fig. 1). Also shown in Fig. 1 are complementation activities of carboxymethylated fragments A and B (sodium dodecyl sulphate was not removed before complementation assay with the crude extract of mutant M15). Duplicate measurements of V_e (elution volume)/ V_0 (void volume) agreed within 0.7%, which would introduce an error of about 2%. The gel-filtration

Fig. 1. Gel filtration on Sephadex G-100 of carboxymethylated β -galactosidase of *E. coli*

The column (150 cm × 2 cm diam.) of Sephadex G-100 was equilibrated at 20°C with the buffer as described in the Experimental section. ●, A_{280} (protein); ○, complementation activity. Abbreviation: CMF, carboxymethylated fraction. The fraction size was 5 ml.

data for marker proteins were found to fit eqn. (1) obtained by the method of least squares:

$$\log M = 6.4041 - 1.5199 (V_e/V_0) \quad (1)$$

The values of the mol.wts. (M) obtained for carboxymethylated fragments A and B by using eqn. (1) were 64300 ± 4300 and 22400 ± 960 respectively.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of proteins was carried out in quadruplicate on 5.6% (w/v) gels. Carboxymethylated fragments A and B were treated with sodium dodecyl sulphate and 2-mercaptoethanol and run on the above gels with marker proteins. A least-squares analysis of the data fitted the equation:

$$\log M = 5.3999 - 1.6488 R_m \quad (2)$$

The plot of $\log M$ versus R_m is linear over the mol.wt. range 16000–130000 and is devoid of any inflexion. The mol.wts. for carboxymethylated fragments A and B calculated from their relative mobilities with eqn. (2) were 64000 ± 4000 and 22100 ± 1500 respectively.

C-Terminal analysis of carboxymethylated fragments A and B

When carboxypeptidase B was used to hydrolyse the carboxymethylated fragments, the C-terminal amino acid appeared to be arginine, 1 mol/mol for

carboxymethylated fragment A and 0.8 mol/mol for carboxymethylated fragment B, with trace amounts of lysine in both fragments.

Molecular weights of the α -acceptors in the extract of mutant M15

The extract of mutant M15 was fractionated on a column of Sepharose 6B; a typical elution profile is shown in Fig. 2. Complementable fractions are outside the regions of the main proteins peaks. Three fractions were obtained from the crude extract of mutant M15 which complemented with carboxymethylated fragment A, and are designated as D_1 , D_2 and D_3 . Three fractions, C_1 , C_2 and C_3 , were obtained from the crude extract of mutant M15 which complemented with carboxymethylated fragment B. These results are summarized in Table 1. For the determination of molecular weights of the complementable fractions and marker proteins, gel-filtration data were obtained under identical conditions. Duplicate determinations of V_e/V_0 agreed within $\pm 0.7\%$, which would introduce an error of about 2% in the determination of the molecular weights of the complementable fractions. The gel-filtration data for marker proteins were found to fit eqn. (3), obtained by the method of least squares:

$$\log M = 7.9685 - 1.3084 (V_e/V_0) \quad (3)$$

The mol.wts. (M) of complementable fractions D_1 ,

Fig. 2. Gel filtration on Sepharose 6B of the crude extract of the mutant M15 and molecular-weight determination of complementable fractions

A column (122 cm × 3 cm diam.) of Sepharose 6B was equilibrated at 20°C with sodium phosphate buffer, pH 7.0. The composition of the buffer is described in the Experimental section. A_{280} (●) and the complementation activity with carboxymethylated fragments A (○) and B (△) are shown.

Table 1. Molecular-weight determinations

Values shown are ±S.E.M. (calculated from eqn. 3), except for the value marked * (for an explanation, see the text).

α-Donors used (and their molecular weights)	Molecular weights of complementable fractions (α-acceptors) from crude extract of mutant M15			Molecular weights of complemented enzymes		
	C ₁	C ₂	C ₃	C _{1a}	C _{2a}	C _{3a}
Carboxymethylated fragment B (22400 ± 960)	507000 ± 11000	262000 ± 5000	123000 ± 2500	525000 ± 13000	525000 ± 13000	525000 ± 1300
	D ₁	D ₂	D ₃	D _{1a}	D _{1b}	D _{1c}
Carboxymethylated fragment A (64300 ± 4300)	507000 ± 11000	253000 ± 4800	63000 ± 1800	D _{2a}	D _{2b}	D _{2c}
				D _{3a}	D _{3b}	D _{3c}
				2000000*	646000 ± 16000	525000 ± 13000

Fig. 3. Gel filtration on Sepharose 6B of the complemented enzymes obtained with carboxymethylated fragment A and complementable fractions D₁(a), D₂(b) and D₃(c) A column (122cm × 3cm diam.) of Sepharose 6B was equilibrated at 20°C with sodium phosphate, pH 7.0. The complementable fractions were incubated with carboxymethylated fragment A. A₂₈₀ (●) and the activities of the complemented enzymes (○) are shown.

D₂, D₃, C₁, C₂ and C₃ calculated with eqn. (3) were 63000 ± 1800, 123000 ± 2500, 253000 ± 4800, 262000 ± 5000 and 507000 ± 11000 respectively.

Molecular weights of complemented enzymes

The fractions C₁, C₂, C₃, D₁, D₂ and D₃ from the column of Sepharose 6B were precipitated with solid

Fig. 4. Gel filtration on Sepharose 6B of the complemented enzymes obtained with carboxymethylated fragment B and complementable fractions C₁ (a), C₂ (b) and C₃ (c) A column (122cm × 3cm diam.) of Sepharose 6B was equilibrated at 20°C with sodium phosphate, pH 7.0. The complementable fractions (see Fig. 3) were incubated with carboxymethylated fragment B. A₂₈₀ (●) and the activities of the complemented enzymes (○) are shown.

(NH₄)₂SO₄ to 50% saturation. The precipitates were dissolved in a 0.1 M-NaH₂PO₄/NaOH buffer, pH 7.0, containing 1 mM-MgCl₂, 0.01 M-2-mercaptoethanol, dialysed in a cold-room, then incubated with car-

Fig. 5. Gel filtration on Sepharose 6B of the complemented enzymes obtained with either carboxymethylated fragments A (a) or B (b) and a crude extract of mutant M15

A column (122 cm $\times$ 3 cm diam.) of Sepharose 6B was equilibrated at 20°C with sodium phosphate buffer, pH 7.0. The crude extract of mutant M15 was incubated with either carboxymethylated fragment A or B as described in the Experimental section. A_{280} (●) and the activities of the complemented enzymes (○) are shown.

boxymethylated fragments A or B. Chromatographic patterns of complemented enzymes of carboxymethylated fragment A with each of the complementable fractions, D₁, D₂ and D₃ are shown in Figs. 3(a), 3(b) and 3(c) respectively. Typical elution profiles of the complemented enzyme activities obtained with carboxymethylated fragment B and complementable fractions C₁, C₂ and C₃ are shown in Figs. 4(a), 4(b) and 4(c) respectively. Complemented enzymes are outside the regions of the main protein peaks. When the total crude extract of mutant M15 was incubated with carboxymethylated fragment A, the complemented enzymes obtained could be separated into three peaks, E₁, E₂ and E₃ (Fig. 5a). However, when the total crude extract of mutant M15 was incubated with carboxymethylated fragment B, only one peak, F, was observed (Fig. 5b). The values of the molecular weights of the complemented enzymes calculated with eqn. (3) are shown in Table 1. The mol.wt. of the complemented enzyme (2000000) calculated from eqn. (3) did not enable us to calculate the standard error. Because a standard with mol.wt. greater than 2000000 was not used, we were not able to calculate a standard error of the mean. The complemented enzyme (mol.wt. 2000000) was eluted in the same fraction as Blue Dextran.

Discussion

The genetic length of the z gene, the structural locus for β -galactosidase, can code for more than 1000 amino acids. This agrees with the mol.wt. of 135000 that has been obtained for a subunit of β -galactosidase and also with the number of amino acid residues that the subunit contains (Sund & Weber, 1963; Steers *et al.*, 1965; Ullmann *et al.*, 1968). Whether this subunit contains one or seven polypeptide chains is still in question. Wallenfels *et al.* (1963) and Steers *et al.* (1965) concluded from physicochemical studies that this subunit contains three to four polypeptide chains each having an average mol.wt. of about 50000. Erickson's (1970) high-speed sedimentation-equilibrium studies showed that β -galactosidase in 6M-guanidinium chloride is heterodispersed with smaller and larger components, yielding an average mol.wt. of 90000. Heterogeneity has also been seen by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Marinkovic *et al.*, 1975).

In addition, Wallenfels & Arens (1960) and Erickson & Steers (1969) analysed purified β -galactosidase and reported the presence of multiple *N*-terminal residues, which suggests the existence of several chains. We obtained arginine as the *C*-terminal amino acid of carboxymethylated fragments A and B. Wallenfels & Gölker (1966) have reported that β -galactosidase has both lysine and arginine as *C*-terminal amino acids, although Korenman *et al.* (1966) and Brown *et al.* (1966) have only identified lysine as the *C*-terminal residue.

We have dissociated carboxymethylated β -galactosidase from *E. coli* into fragments with mol.wts. of 64300 and 22400, and have shown that they could form active β -galactosidase by using α -acceptors from mutant M15. So at least two α -donors are present in β -galactosidase. When we fractionated a crude extract of mutant M15 on a column of Sepharose 6B, four complemented fractions were obtained. Why four complementable fractions were obtained is not yet clearly understood. Whether mutant M15 initially contains four complementable fractions or whether they are an artifact arising from the fractionation procedure is not known. The former possibility is supported by the results of Goldschmidt (1970), who observed protein degradation of nonsense fragments of β -galactosidase *in vivo*.

To understand the nature of the differences between the various forms of complemented enzymes, it is important to know the number of active complementable fractions. The results of the present study support the multichain hypothesis because of the presence of multiple complementable fractions. We obtained three different molecular weights for complemented enzymes formed with carboxymethylated fragment A (Table 1). The complemented enzyme

obtained with carboxymethylated fragment B had the same mol.wt. as one of the complemented enzymes obtained with carboxymethylated fragment A, i.e. 525000. We also obtained complemented enzymes with different molecular weights when an unfractionated crude extract from mutant M15 was incubated with carboxymethylated fragment A. In a similar experiment with carboxymethylated fragment B, only one complemented enzyme was obtained. We do not understand why we obtained multiple complemented enzymes with carboxymethylated fragment A. Perhaps different ratios of α -donor to α -acceptor can yield an active enzyme. In fact, Marchesi *et al.* (1969) have reported that the crude extract of *E. coli* contains multiple forms of active β -galactosidase. The lowest mol.wt. of active wild-type β -galactosidase is reported to be 540000 and the enzyme is composed of four subunits each of mol.wt. 135000 (Sund & Weber, 1963; Steers *et al.*, 1965).

From the results of the experiments discussed above, we assume that the complemented enzymes formed by complementation of mutant M15 *in vitro* with carboxymethylated fragments A and B are a tetramer or multiple forms of active β -galactosidase.

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