A Cyanogen Bromide Fragment of β -Galactosidase from *Escherichia coli* with α -Donor Activity in Complementation of the Enzyme from Mutant M15

By DOBRIVOJE V. MARINKOVIC and JELKA N. MARINKOVIC
The University of Texas Health Science Center at Dallas, Department of Pharmacology,
5325 Harry Hines Boulevard, Dallas, TX 75235, U.S.A.

(Received 4 August 1975)

Aminoethylated β -galactosidase from *Escherichia coli* was cleaved by CNBr. The fragment C_{4a} was purified by gel filtration and ion-exchange chromatography. The molecular weight of the fragment C_{4a} was determined to be 9000 ± 600 . The *N*-terminal amino acid was found to be isoleucine. Qualitative examination of homogeneity was carried out by disc-gel electrophoresis. The fragment C_{4a} was shown to be active as an α donor in complementation of β -galactosidase activity *in vitro* with *E. coli* mutant M15, which has a deletion in the α region of the *z* gene. The molecular weights of completable fractions from mutant M15 were found to be 123000 ± 2500 and 507000 ± 11000 , and of the complemented enzyme 522500 ± 11400 .

Complementation *in vitro* is the restoration of enzyme activity by mixing two enzymically inactive protein fractions. By using various point mutations and deletion mutants of *Escherichia coli* it was first shown by Ullmann *et al.* (1968) that the *z* gene consists of approximately three segments, which produce a polypeptide capable of repairing the β -galactosidase activity in the mutants. The β -galactosidase activity can be recovered when the supernatant from an autoclaved enzyme is mixed with cell extracts of the deletion mutants. The mol.wt. of this fraction was 7400, as determined in sucrose/urea density gradients (Morrison & Zipser, 1970). Lin *et al.* (1970) have reported that a CNBr fragment of wild-type β -galactosidase (mol.wt. about 8000–11000) complemented with an extract of mutant M15. In our earlier study on the complementation *in vitro* of different fragments from β -galactosidase with a crude extract of *E. coli* mutant M15, we found a number of peaks showing β -galactosidase activity (Marinkovic & Tang, 1971). It is possible to observe complementation between fragments of a β -galactosidase molecule, which has been broken chemically, and crude extract prepared from mutant M15. The mutant M15 has no β -galactosidase activity by itself. The present investigation is concerned with the isolation and characterization of CNBr fragment C_{4a} from aminoethylated β -galactosidase of *E. coli*. The amino acid composition and molecular weights were determined. This fragment can be detected by the β -galactosidase activity produced in a complementation reaction.

Experimental

Materials

Chemicals for polyacrylamide-gel electrophoresis were from Canal Industrial Corp., Bethesda, MD,

U.S.A.; sodium dodecyl sulphate and 2-mercaptoethanol were from Fischer, Silver Spring, MD, U.S.A.; Sephadex G-75 and G-50, Sepharose 6B and Blue Dextran 2000 were from Pharmacia, Uppsala, Sweden; phosphocellulose (capacity 0.78 mequiv./g) was from Bio-Rad, Richmond, CA, U.S.A.; CNBr was from Eastman, Rochester, NY, U.S.A.; *o*-nitrophenol β -D-galactoside and isopropyl thio- β -D-galactoside were from Mann Research Laboratories, New York, NY, U.S.A. All other chemicals used were A.R. grade.

Methods

Preparation and characterization of β -galactosidase. *E. coli* K12 3300 strain was obtained from Dr. E. Steers, Jr., National Institutes of Health, Bethesda, MD, U.S.A. The bacteria were grown under the conditions described by Craven *et al.* (1965). The enzyme was purified by the procedures of Hu *et al.* (1959) and Craven *et al.* (1965). The purified β -galactosidase migrated as a single band on polyacrylamide-gel electrophoresis. The specific enzyme activity was 4.48×10^5 units/min per mg of protein. The *N*-terminal residue was threonine, as determined by the dansyl method (Gray & Hartley, 1963).

Preparation of crude extract from *E. coli* mutant M15. Mutant E706/M15 was obtained from Dr. J. R. Beckwith, Harvard Medical School, Boston, MA, U.S.A., and was used as the α acceptor in complementation experiments. The mutant was grown in the mineral medium 73 as described by Herzberg (1959) with 0.5 mM-isopropyl thio- β -D-galactoside. The crude extract of mutant M15 was prepared in an Aminco Press at 80–100 MPa (800–1000 atm) in 0.1 M-sodium phosphate buffer, pH 7.20, containing 1 mM-MgSO₄, 0.01 M-NaCl, 2 mM-EDTA, 0.2 mM-

MnSO₄ and 0.1M-2-mercaptoethanol. The supernatants obtained after centrifugation of the homogenates in a Sorvall centrifuge for 50 min at 4°C and 20000g were used in all the experiments. Protein concentration in the extract was assayed by the microbiuret method (Goa, 1953) and was 10–30 mg/ml.

Reduction and alkylation of β -galactosidase. β -Galactosidase (2g) was dissolved in 0.05M-Tris/acetate buffer, pH9.0, containing 65mM-EDTA and 8M-urea, to a concentration of 10mg of protein/ml. 2-Mercaptoethanol was then added to a final concentration of 1mM and the solution was incubated at room temperature (20°C) for 16h. After the incubation, cysteamine was introduced in a 100-fold molar excess over the 2-mercaptoethanol. The reaction mixture was adjusted to pH9.50 with 1M-NH₃ and then incubated at 37°C for 36h. The final product was dialysed for 3 days against water at pH9.0 adjusted with 1M-NH₃.

Cyanogen bromide reaction. The freeze-dried, reduced cysteamine-ethylated β -galactosidase was treated with CNBr by the procedure described by Steers *et al.* (1965). The protein was dissolved in 70% (v/v) formic acid at a concentration of 3 mg/ml. A 50-fold excess of crystalline CNBr was added and the reaction was allowed to proceed at room temperature (20°C) overnight. The reaction mixture was diluted 10-fold with water and freeze-dried.

Gel filtration. The CNBr fragments were separated by using a column (105 cm $\times$ 5 cm diameter) of Sephadex G-75 equilibrated in 0.1M-ammonium acetate buffer, pH9.0. CNBr fragments (2g) were dissolved in 0.1M-ammonium acetate, pH9.0, the solution was adjusted to pH9.3 with 1M-NH₃, and 1.5g of the soluble material was applied to the Sephadex column. Fractions (10ml) were collected at a flow rate of 40 ml/h.

Ion-exchange chromatography. A phosphocellulose column (31 cm $\times$ 1.5 cm diameter) was equilibrated with 1mM-sodium acetate/acetic acid, pH3.8, containing 2M-urea. A weighed sample of CNBr fragments was dissolved in 4ml of 1mM-sodium acetate buffer, pH3.8, containing 8M-urea. The solution was kept overnight at room temperature (20°C) and diluted to 20ml with 1mM-sodium acetate buffer, pH3.8. The material was eluted from the column at room temperature by using equilibrated buffer followed by 1mM-sodium acetate buffer, pH3.80, containing 0.1M-NaCl and 4M-urea, and finally by 0.01M-sodium acetate buffer, pH3.80, containing 0.5M-NaCl and 8M-urea. The volume of each buffer was 200ml. Fractions (5ml) were collected at 25 ml/h. Samples (0.1 ml) of each fraction were assayed for complementation activity with crude extract of mutant M15. The NaCl gradient was followed by measuring the conductivity of the fractions with a conductivity-measuring bridge,

type CDM-2 (Radiometer, Copenhagen, Denmark). The fractions representing given peaks were combined, freeze-dried, redissolved in 5 ml of 25% (v/v) acetic acid, desalted at room temperature on a column (30 cm $\times$ 3 cm diameter) of Sephadex G-25, equilibrated in 25% (v/v) acetic acid and freeze-dried again.

Molecular-weight determination of CNBr fragment. The molecular size of the CNBr fragment was investigated by using a column (150 cm $\times$ 2 cm diameter) of Sephadex G-50 equilibrated in 0.1M-sodium acetate buffer, pH7.20, containing 0.1M-NaCl. The column was calibrated with the following standards (mol.wts. in parentheses): trypsin (23 800); lysozyme (14 400), from Worthington Biochemical Corp., Freehold, NJ, U.S.A.; ribonuclease (12 500), from Sigma, St. Louis, MO, U.S.A.; bovine insulin (6000), β -chain of insulin (3000), both from Mann Research Division at Becton, Dickinson Co., New York, NY, U.S.A. Fractions (4ml) were collected at 15 ml/h. A plot of the elution volumes of the standards against the logarithms of the molecular weights was used to estimate the molecular weight of the CNBr fragment.

Amino acid analysis. Samples (about 1 mg) of protein were hydrolysed in 0.2 ml of constant-boiling HCl in evacuated tubes at 105°C for 20, 40, and 72 h. The hydrolysates were evaporated *in vacuo* at room temperature and analysed with a Beckman Spinco amino acid analyser by the method of Spackman *et al.* (1958). Norleucine was added as standard. A portion of dry hydrolysate was oxidized by performic acid by the procedure of Hirs (1956) for the determination of half-cysteine as cysteic acid and cysteamine as taurine.

Gel electrophoresis. Electrophoretic study of the fragments was carried out as described in the manual of Canal Industrial Corp.; 7.5% (w/v) polyacrylamide gel (6 mm $\times$ 120 mm) was prepared in 0.75M-Tris/HCl, pH8.9, measured at room temperature (20°C). The sample was dissolved in the electrode buffer, 10mM-Tris/glycine, pH8.5, and was then applied for electrophoresis (10 μ l, containing 0.1 mg of protein). A constant current of 3 mA per tube was applied and the electrophoresis was carried out at 4°C for 3 h. After electrophoresis the gel was fixed in 12.5% (w/v) trichloroacetic acid for 1 h. Proteins were stained with 0.12% Coomassie Brilliant Blue R-250 in water for 5 h, and gels were destained with 7% (v/v) acetic acid.

Gel electrophoresis in the presence of sodium dodecyl sulphate. The method of Shapiro *et al.* (1967) was followed with slight modification; 10% (w/v) polyacrylamide gel (6 mm $\times$ 120 mm) was prepared in 0.05M-sodium phosphate buffer, pH7.20, measured at room temperature (20°C). After polymerization, the gel was pre-electrophoresed for 2 h with a constant current of 6 mA per tube

at room temperature. A 10 μ l sample containing about 0.1mg of protein was added at the top of the gel with 10 μ l of 40% (w/v) sucrose. Electrophoresis was carried out with a constant current of 3mA per tube for the first 30min, and then with 6mA per tube for 2h. The gel was fixed, stained and destained as described above. The gels were calibrated with the following standards (mol.wts. in parentheses): bovine haemoglobin, α and β chains (16000), from Sigma; cytochrome *c* (13000), from Nutritional Biochemical Corp., Cleveland, OH, U.S.A.; lysozyme (14400), from Worthington; α and β chains of insulin (taken as 3000) from Mann Research Division at Becton, Dickinson. A plot of the mobility of the standards against the molecular weights was used to estimate the molecular weight of the CNBr fragment.

Gel electrophoresis in the presence of 8M-urea. Electrophoretic study of the fragment was carried out by the method of Gesteland & Staehelin (1967), except that alkaline, instead of acidic, conditions were used. A 5% (w/v) separating polyacrylamide gel (6mm $\times$ 120mm) was prepared in 0.12M-Tris/HCl, pH9.0, measured at room temperature. A sample of the fragment was dissolved in aq. 8M-urea, and then applied for electrophoresis (20 μ l, containing 0.1mg of protein). Electrophoresis was carried out at 4°C for 1.5h at 3mA per tube with 5mM-Tris/glycine, pH8.5. The gel was fixed, stained and destained as described above.

N-Terminal amino acid analysis. The fragment C_{4a} (1mg) was treated with dansyl chloride in 4M-urea/0.1M-sodium phosphate, pH9.4, as suggested by Gros & Labouesse (1969). The reaction mixture was kept at room temperature for 30min. The total volume of the mixture was 0.75ml. The sample was passed through a Sephadex G-10 column (21cm $\times$ 0.9cm diameter). The dansylated fragment C_{4a} is easily separated from materials of low molecular weight since it can be made visible by illuminating the column with a u.v. lamp. The sample was freeze-dried. After hydrolysis in constant-boiling HCl (0.2ml) for 16h at 105°C in a sealed tube the hydrolysate was dried *in vacuo* over NaOH and H₂SO₄. A dry sample was dissolved in acetone/acetic acid (3:2, v/v) and then subjected to polyamide-layer chromatography as described by Woods & Wang (1967). The fluorescent dansyl-amino acid was observed under u.v. light.

Molecular-weight determination of the complementable fractions. The clear supernatant from the crude extract of mutant M15 was applied to a column (122cm $\times$ 3cm diameter) of Sepharose 6B, which had been equilibrated with 0.05M-sodium phosphate, pH7.0, containing 1mM-MgCl₂, 2mM-EDTA and 0.01M-2-mercaptoethanol. The same buffer was used for elution at a flow rate of 40ml/h, and fractions (4.8ml) were collected. The comple-

mentable fractions for each tube were tested with the CNBr fragment. The peak fractions (*a* and *b*, Fig. 5) were pooled separately and precipitated with solid (NH₄)₂SO₄. The molecular weights of the complementable fractions and complemented enzymes were investigated by using the above column. The column was calibrated with the following standards (mol.wts. in parentheses): β -galactosidase (108000 and 540000), purified ourselves from *E. coli* K12 3300; porcine thyroglobulin (660000); bovine albumin (69000), from Sigma; human γ -globulin (fraction III, 157000), from Mann Research Division at Becton, Dickinson; ovalbumin (45000), from Pentex, Kankakee, IL, U.S.A. A plot of the elution volumes of the standards against the logarithms of the molecular weights were used to estimate the molecular weights of complementable fractions and complemented enzymes.

Ammonium sulphate precipitation. Solid (NH₄)₂SO₄ was added to complementable fractions *a* and *b* at 4°C to 50% saturation. The precipitates were collected by centrifugation and then dissolved in 0.05M-sodium phosphate, pH7.0, containing 1mM-MgCl₂, 2mM-EDTA and 0.1M-2-mercaptoethanol. The solutions *a* and *b* (see above) were dialysed against the same buffer in a cold-room for 2 days. After this, a small amount of insoluble material was removed by centrifugation. The fractions *a* and *b* were then incubated with fragment C_{4a} to estimate the molecular weights of complemented enzymes.

Complementation assay. For the assay of complementation activity the methods of Pardee *et al.* (1959) were followed. *o*-Nitrophenyl β -D-galactoside was used as substrate. Each tube contained 100 μ l of the incubated mixture, 1.5ml of 0.1M-sodium phosphate, pH7.0, containing 1mM-MgCl₂ and 0.1M-2-mercaptoethanol, and 0.25ml of substrate solution (75mg/100ml of sodium phosphate, pH7.0) in a total volume of 1.85ml. The blank, containing the same amounts of buffer and substrate but with water instead of the test solution, was treated identically. Incubation was carried out at 37°C for 16h. At the end of the incubation, the reactions were stopped by adding 0.25ml of 1M-Na₂CO₃. The E_{420} of the samples was read in a Perkin-Elmer spectrophotometer. Enzyme activity was expressed in units, as described by Pardee *et al.* (1959).

Incubation to form complemented enzymes. The reaction mixtures for complemented enzymes were composed of 3.5ml of 0.1M-sodium phosphate, pH7.0 (containing 1mM-MgCl₂ and 0.01M-2-mercaptoethanol), 2mg of CNBr fragment C_{4a} dissolved in 1ml of 0.02M-Tris/HCl, pH7.20 (containing 0.01M-EDTA, 0.01M-NaCl and 0.1M-2-mercaptoethanol) and 2ml of fractions *a* or *b*, which were prepared in 0.05M-sodium phosphate, pH7.0 (containing 1mM-MgCl₂, 2mM-EDTA and 0.01M-2-mercaptoethanol). Incubation was carried out at

37°C for 20h. At the end of incubation the samples were centrifuged for 20min at 4°C at 20000g. The supernatants were concentrated to 2ml with Sephadex G-200 and then applied to a Sepharose 6B column (122cm×3cm diameter) at a flow rate of 40ml/h, and fractions (5ml) were collected.

Results and Discussion

Gel filtration

Gel filtration on Sephadex G-75 demonstrated that peptides of different molecular weights were obtained by CNBr cleavage of ethylated β -galactosidase. More than 75% of the peptides were soluble in ammonium acetate buffer, pH9.3. The results are shown in Fig. 1. The fragments were separated into four peaks, A, B, C and D. Peak-C material showed complementation activity with mutant M15. Fractions from peak C were pooled and freeze-dried, and this peak-C material was then fractionated on a Sephadex G-50 column as shown in Fig. 2. Complementation activity was found in the peak C₁. Fractions from the Sephadex G-50 column containing complementation activity were pooled and freeze-dried.

Ion-exchange chromatography of CNBr fragments

To investigate whether distinct acidic and basic α -donor components existed in aminoethylated β -galactosidase, peak-C₁ material was analysed by ion-exchange chromatography on phosphocellulose

Fig. 1. Gel filtration on Sephadex G-75 of the CNBr fragments from aminoethylated β -galactosidase of *E. coli*

The column (105cm×5cm diameter) of Sephadex G-75 was equilibrated at 20°C with 0.1M-ammonium acetate, pH9.0. A CNBr digest of aminoethylated β -galactosidase (about 1.5g of protein in 10ml of appropriate buffer, pH9.3) was added to the column of Sephadex G-75 and the effluent examined spectrophotometrically at 280nm. Fractions (10ml) were collected and pooled, as indicated by bars, as A, B, C and D.

(Fig. 3). The peak containing complementation activity was eluted near the end of the NaCl gradient. The unadsorbed acidic material from peak C₁ was eluted by washing the column with starting buffer. Each peak from this phosphocellulose column chromatography was collected, freeze-dried and desalted as described in the Experimental section.

Homogeneity of C_{4a} fragment

The homogeneity of the isolated CNBr fragment C_{4a} was confirmed by disc-gel electrophoresis at pH8.5. The isolated fraction gave a single sharp band. A single more diffuse band was also obtained after disc-gel electrophoresis in the presence of sodium dodecyl sulphate. Electrophoresis with 8M-urea gave a single tight band of CNBr fragment C_{4a}. The gel-chromatographic results below also demonstrate that the CNBr fragment C_{4a} was obtained essentially in homogeneous form.

Determination of the molecular weight of the fragment C_{4a}

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of proteins was generally carried out in triplicate on 10% gels, by using the same batch of

Fig. 2. Gel filtration on Sephadex G-50 of the CNBr fragments of peak C from Sephadex G-75

The column (155cm×2cm diameter) of Sephadex G-50 was equilibrated at 20°C with 0.1M-ammonium acetate, pH9.0. The peak C from Sephadex G-75 (about 290mg of material in 3ml of appropriate buffer) was added to a column of Sephadex G-50. E_{280} (●) and complementation activity (○) of the effluent were recorded. Fractions (approx. 5ml) were collected and pooled as indicated by bars as C₁ and C₂.

Fig. 3. Phosphocellulose chromatography of the CNBr fragments of peak C₂ from Sephadex G-50

Columns (31 cm × 1.5 cm diameter) of phosphocellulose were equilibrated at 20°C with sodium acetate, pH 3.8, containing 2 M-urea. The peak C₁ from the Sephadex G-50 column (about 120 mg of material in 20 ml of appropriate buffer) was added to the column of phosphocellulose, washed with the same buffer and the fragments were eluted with the salt and urea gradient. See the Experimental section for further details. E₂₈₀ (●) and complementation activity (○) of the effluent were recorded. ▲, NaCl concentration.

Fig. 4. Calibration curve for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of proteins

Proteins were reduced and denatured in 1% (w/v) sodium dodecyl sulphate and 1% (v/v) 2-mercaptoethanol. After incubation for 3 h at 37°C, about 0.1 mg of protein was applied to a polyacrylamide gel (10%, w/v; 0.6 cm × 12 cm) containing 0.1% sodium dodecyl sulphate and electrophoresed in 0.1 M-sodium phosphate, pH 7.10, for 30 min with a current of 3 mA/gel, and then at 6 mA/gel for 2 h. The gels were stained with Coomassie Brilliant Blue R-250 and destained with 7% (v/v) acetic acid. The molecular weights of the proteins (in parentheses) are plotted against R_m values: 1, β chain of insulin (taken as 3000); 2, α and β chains of insulin (taken as 3000); 3, CNBr-fragment C_{4a} (8100); 4, cytochrome c (13 000); 5, lysozyme (14 400); 6, α and β chains of bovine haemoglobin (16 000).

Table 1. Amino acid composition of fragment C_{4a} from aminoethylated β-galactosidase

The number of residues per molecule was the average (±S.E.M.) from three analyses after 20, 40 and 72 h of hydrolysis, except for the values of threonine and serine, which were extrapolated to zero time of hydrolysis. The calculation is based on a mol.wt. 9000. Half-cysteine and cysteamine were determined after performic acid oxidation as cysteic acid and taurine. The total number of residues is 77, equivalent to a mol.wt. of 8700.

Amino acid	No. of residues per molecule	Nearest integral
Lysine	3.12 ± 0.25	3
Histidine	4.58 ± 0.32	5
Arginine	8.67 ± 0.26	9
Aspartic acid	8.54 ± 0.20	9
Threonine	3.80	4
Serine	5.08	5
Glutamic acid	8.62 ± 0.29	9
Proline	3.13 ± 0.05	3
Glycine	5.32 ± 0.17	5
Alanine	4.70 ± 0.11	5
Half-cysteine	0.87 ± 0.01	1
Valine	3.75 ± 0.17	4
Homoserine	0.89 ± 0.02	1
Isoleucine	3.02 ± 0.11	3
Leucine	7.20 ± 0.21	7
Tyrosine	1.32 ± 0.08	1
Phenylalanine	2.35 ± 0.13	2
Cysteamine	0.85 ± 0.02	1

solution of acrylamide and methylene-bisacrylamide. A least-squares analysis of the data fitted the equation:

$$\log M = 7.3499 - 4.8473 R_m \quad (1)$$

where M denotes the mol.wt. of the protein polypeptide and R_m is linear. The results are shown in Fig. 4 and indicate a mol.wt. of 8100 ± 670 . The mol.wt. of the fragment C_{4a} , calculated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis is about 11% lower than that obtained by filtration behaviour.

Gel chromatography

Data for marker proteins and CNBr fragment C_{4a} were obtained under identical conditions. Duplicate or triplicate determinations of V/V_0 agreed within $\pm 0.7\%$, which would introduce an error of about 2%. The gel-filtration data for marker proteins were found to fit the following equation, obtained by the method of least squares:

$$\log M = 5.8247 - 1.1046(V/V_0) \quad (2)$$

The value of the mol.wt. (M) of the fragment C_{4a} determined from the equation is 9000 ± 600 . The mol.wt. of the fragment C_{4a} , calculated on the basis of

its amino acid composition, is 8700 (see Table 1) and is only about 3.3% lower than that obtained by gel filtration. We are inclined to accept the 9000 value as reliable and to neglect the result obtained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, because this method becomes unreliable at low molecular weights. It is possible that the fragment C_{4a} and that described by Lin *et al.* (1970) are the same. Marinkovic & Tang (1971) showed complementation activity in fraction B (Fig. 1) and found a mol.wt. of 14000 for the α donor. We have been able to separate fraction B into five subfractions, of which three have complementation activity, but we have not been able to establish the homogeneity of any of them. Treatment of proteins with CNBr sometimes converts methionine residues into homoserine with only partial cleavage of the polypeptide chain (see, e.g., DeLange, 1970), and the various fractions with complementation activity could still all contain the same active region, and differ only by being extended in one or both directions.

Amino acid analysis

The amino acid composition of the CNBr fragment C_{4a} is shown in Table 1. The analysis shows

Fig. 5. Gel filtration on Sepharose 6B of the crude extract of the mutant M15 and molecular-weight determination of complementation fractions

A column (122cm $\times$ 3cm diameter) of Sepharose 6B was equilibrated at 20°C with sodium phosphate, pH 7.0. The composition of the buffer is described in the Experimental section. The crude extract of mutant M15 (about 315 mg of protein in 10 ml of appropriate buffer) was added to the column of Sepharose 6B, eluted with the same buffer and the complementation fractions were assayed in the effluents as described in the Experimental section. E_{280} (●) and the complementation activity (○) are recorded.

that 94% of the methionine residues were converted into homoserine with CNBr in 70% (v/v) formic acid as the solvent. It was shown that oxidation by performic acid of cysteamine to taurine, and of cystine to cysteic acid, occurs in the ratio 1:1. It was assumed that the CNBr fragments (with the exception of the C-terminal one) would contain one residue of homoserine. The mol.wt. of the C_{4a} fragment calculated on the basis of its amino acid composition is 8700.

N-Terminal analysis of fragment C_{4a}

By using the dansyl method, the *N*-terminal amino acid appeared to be isoleucine with trace amounts of leucine and serine.

Fig. 6. Gel filtration of Sepharose 6B of the complemented enzymes obtained from complementable fractions *a* or *b* and CNBr fragment C_{4a} .

A column (122cm $\times$ 3cm diameter) of Sepharose 6B was equilibrated at 20°C with sodium phosphate, pH 7.0. The complementable fractions (about 30mg of protein *a* and 25 mg of protein *b*) were incubated with 2mg of CNBr-fragment C_{4a} . For further details, see the Experimental section. Concentrated solutions (2ml) of complemented enzymes were added to the column of Sepharose 6B, which was then eluted with the same buffer and complemented enzymes were assayed in the effluents as described in the Experimental section. E_{280} (●) and the activity of the complemented enzymes (○) are recorded.

Molecular weight of the α acceptor in the extract of mutant M15

For the determination of molecular weights of the complementable fractions and marker proteins, gel-filtration data were obtained under identical conditions. Duplicate determination of V/V_0 agreed within $\pm 0.7\%$, which would introduce an error of about 2% in the determination of molecular weight of the complementable fractions. The gel-filtration data for marker proteins were found to fit the following equation, obtained by the method of least squares:

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

The mol.wts. (M) of complementable fractions calculated from the equation are 123000 ± 2500 and 507000 ± 11000 . The extract of mutant M15 was fractionated on the Sepharose 6B column; a typical elution profile is shown in Fig. 5. Two fractions were obtained from the crude extract of mutant M15 that complemented with CNBr fragment C_{4a} . Complementation of the two fractions of M15 with fragments C_1 and C_{4a} gave enzyme activities of 39000, 25400 and 2800 units/mg of protein respectively. The high sensitivity of the assay for β -galactosidase activity makes very small amounts of CNBr fragment C_{4a} easily detectable.

This complementation shows high affinity and specificity; the CNBr fragment C_{4a} was able to reassociate even at high dilution and in the presence of the other proteins. Marinkovic & Tang (1971) reported that five different α acceptors are present in the mutant M15. The presence of two complementable fractions in the extract of mutant M15 is not yet certain. It is possible that the monomer of mol.wt. 123000 is a product of dissociation of the defective β -galactosidase from mutant M15. This possibility is supported by Goldschmidt (1970), who observed protein degradation of nonsense mutation of β -galactosidase. The purified CNBr fragment C_{4a} contained one aminoethyl cysteine residue, indicating that a thiol group or aminoethyl cysteine is not required for complementation activity. Ullmann & Perrin (1970) reported that carboxymethylated β -galactosidase completely lost enzymic activity in 8M-urea. However, after removal of urea by dialysis the α -donor activity was still present, indicating that either the α donor did not contain thiol groups or that thiol groups were not required for complementation.

Molecular weight of complemented enzyme

The value of the mol.wt. of the complemented enzyme is, from eqn. (3), 522500 ± 11400 , which is within an experimental error of about 2%. The molecular weights of complemented enzyme from the

Fig. 7. Model proposed for the α -complemented enzyme of mutant M15 with CNBr-fragment C_{4a}.

The mutant M15 protein is the acceptor and CNBr-fragment C_{4a} is the donor.

two fractions of mutant M15 complementable with CNBr fragment C_{4a} were determined from Sepharose 6B chromatography as shown in Fig. 6. A mol.wt. of 522500 was observed for the complemented enzyme when either of the two complementable fractions was used. These findings correspond to the α acceptor of mol.wt. 123000 having associated with one α -donor CNBr fragment, C_{4a}, and four of these protomers having aggregated to form the active tetramer, whereas one α acceptor of mol.wt. 507000 appears to have the ability to associate with four donors to form the tetramer. It can thus be proposed that the complemented enzyme synthesized by complementation of mutant M15 *in vitro* with CNBr fragment C_{4a} is a tetramer (Fig. 7). It is possible that the tertiary structure of the complemented enzyme is very similar to the structure of the corresponding regions in the wild-type enzyme.

We thank Dr. J. Martinovic and Dr. M. Judy for their careful reading of this manuscript and valuable suggestions, and also Mrs. Esther Ruiz for its preparation. This work was supported in part by NIH grant no. 7 ROL GM20947-01.

References

- Craven, G. R., Steers, E., Jr. & Anfinsen, C. B. (1965) *J. Biol. Chem.* **240**, 2468–2477
- DeLange, R. J. (1970) *J. Biol. Chem.* **245**, 907–916
- Gesteland, F. R. & Staehelin, T. (1967) *J. Mol. Biol.* **24**, 149–155
- Goa, J. (1953) *Scand. J. Clin. Lab. Invest.* **5**, 218–222
- Goldschmidt, P. (1970) *Nature (London)* **228**, 1151–1154
- Gray, W. R. & Hartley, B. S. (1963) *Biochem. J.* **89**, 379–380
- Gros, C. & Labouesse, B. (1969) *Eur. J. Biochem.* **7**, 463–470
- Herzberg, L. A. (1959) *Biochim. Biophys. Acta* **31**, 525–538
- Hirs, C. H. W. (1956) *J. Biol. Chem.* **219**, 611–621
- Hu, A. S. L., Wolfe, G. R. & Reithel, F. J. (1959) *Arch. Biochem. Biophys.* **81**, 500–507
- Lin, S., Villarejo, M. & Zabin, I. (1970) *Biochem. Biophys. Res. Commun.* **40**, 249–254
- Marinkovic, D. V. & Tang, J. (1971) *Biochem. Biophys. Res. Commun.* **45**, 1288–1293
- Morrison, L. S. & Zipser, D. (1970) *J. Mol. Biol.* **50**, 359–371
- Pardee, A. B., Jacob, F. & Monod, J. (1959) *J. Mol. Biol.* **1**, 165–178
- Shapiro, A. L., Vinuela, E. & Maizee, J. V. (1967) *Biochem. Biophys. Res. Commun.* **28**, 815–820
- Spackman, D. H., Stein, W. H. & Moore, S. (1958) *Anal. Chem.* **30**, 1190–1206
- Steers, E., Jr., Craven, G. R., Anfinsen, C. B. & Bethune, J. L. (1965) *J. Biol. Chem.* **240**, 2478–2484
- Ullmann, A. & Perrin, D. (1970) in *The Lactose Operon* (Beckwith, J. R. & Zipser, D., eds.), pp. 143–172, Cold Spring Harbor Laboratory, Cold Spring Harbor
- Ullman, A., Jacob, F. & Monod, J. (1968) *J. Mol. Biol.* **32**, 1–13
- Woods, R. K. & Wang, T. K. (1967) *Biochim. Biophys. Acta* **133**, 369–370