β-Galactosidase α Complementation: Properties of the Complemented Enzyme and Mechanism of the Complementation Reaction[†]

Keith E. Langley[‡] and Irving Zabin*

ABSTRACT: Intracistronic α complementation involving *Escherichia coli* β-galactosidase occurs between the cyanogen bromide peptide CB2, derived from residues 3–92 of β-galactosidase (Langley, K. E., Fowler, A. V., and Zabin, I. (1975), *J. Biol. Chem. 250*, 2587), and the defective β-galactosidase from the Z-deletion mutant strain M15. The M15 protein, a dimer, lacks residues 11–41 of β-galactosidase (Langley, K. E., Villarejo, M. R., Fowler, A. V., Zamenhof, P. J., and Zabin, I. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72*, 1254). The complemented enzyme formed from purified components has a molecular weight of 533 000 ± 25 000, is therefore tetrameric, and has a probable stoichiometry of 1 CB2:1 M15 monomer. The complemented enzyme has the same K_m for substrate as wild type enzyme, but is less stable to heat or urea treatment. The overall equilibrium constant for the complementation reaction is approximately $1-2 \times 10^9$ M^{-1}. Initial velocity studies indicate saturation kinetics when either component is fixed and limiting, with an apparent K_d of about 10^{-6} M. A first-order rate constant of 0.05–0.1 min^{-1} was estimated. The kinetics favor a model of rapid complex formation, followed by slow conformational change, as the mechanism of activation. Ultraviolet difference spectroscopy indicated an increased absorbance in the 290–300 nm region as a result of the complementation reaction. The kinetics of the increase suggest that two processes, one rapid and the other slower, could be responsible. The temperature dependence of complementation (E_a ~24 000 cal) is also consistent with the rate-determining step being a conformational change.

α-Complementation, an example of intracistronic complementation involving fragments of *Escherichia coli* β-galactosidase (EC 3.2.1.23) (Ullmann et al., 1967), can occur between a cyanogen bromide digest of the wild-type enzyme and extracts of the *lac* Z operator-proximal deletion mutant strain M15 (Lin et al., 1970). Recently, we have isolated the α-donor cyanogen bromide peptide (CB2) and have shown by sequence determination that it represents residues 3–92 of β-galactosidase, or approximately $^1/_{13}$ of the intact chain (Langley et al., 1975a). This work contributes to the sequence determination of β-galactosidase, which is in progress (Fowler and Zabin, 1970; Fowler, 1972b; Zabin and Fowler, 1972). In addition, we have isolated and characterized the α-acceptor M15 deletion mutant protein (Langley et al., 1975b). The protein is dimeric under conditions where wild-type β-galactosidase is tetrameric, and residues 11–41 of β-galactosidase are missing as a result of the deletion. The molecular weight of the M15 protein monomer is approximately 132 000.

Since the segment missing from M15 protein is included within CB2, it is clear that activity is restored in the complementation because CB2 supplies the residues missing from the α acceptor. Using the purified components, we have now focused on the complementation reaction. We report studies on the affinity between CB2 and 15 protein and on the stoichiometry of their interaction. In addition, we have studied properties of the complemented enzyme in comparison to wild-type β-galactosidase and, using kinetic and physical chemical approaches, have investigated the mechanism of the interaction leading to the active complex.

Materials and Methods

Materials. β-Mercaptoethanol, EDTA,[1] and urea were obtained from Matheson, Coleman, and Bell. Urea was always prepared as a 10 M solution and deionized by passage through a mixed bed ion-exchange resin (Bio-Rad AG 501-X8(D)). EGTA was from Sigma and dithioerythritol from Pierce Chemical Co. Bio-Gel A-1.5 m gel filtration matrix was purchased from Bio-Rad, and Spectrapor 3 dialysis membrane from Spectrum Medical Industries. BSA was obtained as a 22% solution from Armour Pharmaceutical Co. Before use, it was dialyzed extensively against buffer C, then against buffer A. [2-^{3}H]Iodoacetic acid (96 mCi/mmol) was supplied by Amersham/Searle.

Buffer Designations. The following buffer designations will be used: buffer A, 0.1 M sodium phosphate, 10^{-3} M MgSO$_4$, 0.05 M β-mercaptoethanol, pH 7; buffer B, 0.1 M sodium phosphate, 10^{-3} M MgSO$_4$, 2×10^{-4} M MnSO$_4$, 0.05 M β-mercaptoethanol, pH 7; buffer C, 0.1 M sodium phosphate, 10^{-3} M MgSO$_4$, 10^{-3} M EGTA, pH 7. The suffix, –BSA, indicates that 0.5 mg/ml of BSA is included in the buffer.

Isolation of β-Galactosidase, M15 Protein, and CB2; Preparation of Protein Solutions. The procedures for purification of β-galactosidase (Fowler, 1972a), M15 protein (Langley et al., 1975b), and CB2 (Langley et al., 1975a) have been described previously.

β-Galactosidase preparations were freed of multimers higher than tetramer by passage through a column of Bio-Gel A-1.5 m.

Preparations of M15 protein contained very low levels of β-galactosidase activity (about 0.01% of wild type). The purified protein, dissolved in buffer A, 10% glycerol, was stored

at -40 °C after freezing in a dry ice-ethanol bath. Even when subjected to frequent freezing and thawing, the protein stored in this way showed no loss in α-acceptor activity for at least 6 months. On the basis of sedimentation equilibrium (Langley et al., 1975b) and gel filtration on Bio-Gel A-1.5 m, the M15 protein appeared to be a homogeneous, unaggregated dimer when dissolved in the buffers used. It had a specific activity of 400 000 units/mg in the α-acceptor assay, or about 90% of the value for wild-type β-galactosidase.

Buffered CB2 solutions were prepared as follows. CB2, usually in 30% acetic acid, was dried by rotary evaporation, and then dissolved in 0.1 M sodium phosphate solution at pH 10–11. The solution was then immediately dialyzed in Spectrapor 3 membrane against the desired buffer.

·As judged by gel filtration with a column of Sephadex G-75 (superfine), the CB2 solutions prepared in this manner sometimes contained aggregated forms. These could be disrupted with 8 M urea and did not appear to re-form. Therefore, CB2 solutions for use in experiments where the aggregation was undesirable was pretreated by dialysis against 0.1 M sodium phosphate, 8 M urea, pH 7 (about 10 h at 4 °C), followed by dialysis against the desired buffer. All CB2 solutions were also routinely centrifuged for 1 h at 45 000g before use in kinetic and physical chemical experiments.

[CM-^{3}H]CB2 was isolated from a cyanogen bromide digest of [CM-^{3}H]β-galactosidase, prepared by reaction with [2-^{3}H]iodoacetic acid (Crestfield et al., 1963).

Protein concentrations were measured spectrophotometrically, using $\epsilon_{280}^{0.1\%} = 2.09$ (Craven et al., 1965) for β-galactosidase, 2.03 for M15 protein, and 3.1 for CB2. The latter two values were determined by amino acid analysis of solutions with known A_{280}. The three $\epsilon_{280}^{0.1\%}$ values correspond to ϵ_{280}^{M} values of 282 000, 268 000, and 32 000 (respectively). All molarity concentrations of M15 protein and β-galactosidase are given in terms of the protein monomer.

Time Course Studies of Complementation. For all studies in which the time course of complementation was followed, CB2 and M15 protein, each dissolved in phosphate buffer, were combined to give the desired concentrations. Unless indicated otherwise, the final buffer system was always buffer A–BSA and the incubation temperature 28 °C during complementation. To follow the appearance of activity, aliquots of size appropriate for 0.5–5 min β-galactosidase assay time were removed, diluted into buffer B, and assayed immediately. The dilutions made effectively stopped the complementation. The method of Horiuchi et al. (1962) was used for the β-galactosidase assays.

The presence of 0.5 mg/ml of BSA in the reaction mixtures improved the efficiency (by as much as 35%) and reproducibility of the results, especially at low concentrations, without affecting the kinetics of complementation.

Complementation v_0 Studies. v_0 measurements were made in a Cary 16 spectrophotometer with recorder and thermostated cell holder. All measurements were at 28 °C in buffer A–BSA containing 2.6×10^{-3} M ONPG. The components were mixed, placed in the spectrophotometer cell, and the recorder started within 20 s after mixing. A_{420} was followed as a function of time, with the recorder set at the scale most appropriate for slope estimates. The slopes, representing $\Delta A_{420}/\Delta t$, were converted to EU of β-galactosidase activity. The conversion included factors to correct for the volume and pH differences from the usual β-galactosidase assay. (Corrected ϵ_{420}^{M} for o-nitrophenol = 2160.) v_0 was then estimated from plots of EU formed/ml vs. time, and was expressed as EU/ml min. These plots sometimes showed a slight lag period
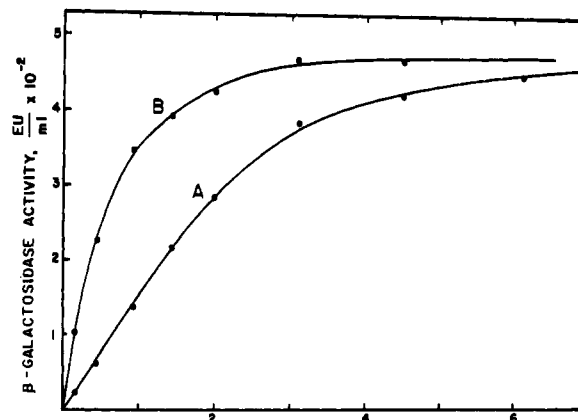

FIGURE 1: Complementation time course. Curve A, M15 protein = 10^{-8} M, CB2 = 10^{-7} M. Curve B, M15 protein = 10^{-8} M, CB2 = 10^{-6} M.

before reaching a linear region and then beginning to level off. v_0 was always estimated from the linear portion.

Ultraviolet Difference Spectroscopy. Spectra were taken at room temperature in a Cary Model 14 recording spectrophotometer using the 0.1 scale and automatic slit-control mode. To determine the uv difference of mixed M15 protein and CB2 relative to unmixed, the tandem cell technique (Herskovits and Laskowski, 1962) was applied. CB2 and M15 protein solutions, in buffer A or C, were placed separately in the compartments of reference and sample cells. The path length of each compartment was 4 mm. Baseline (t_0) spectra were taken with this configuration. The CB2 and M15 proteins in the sample cells were then mixed by removing each from the cell, combining equal weights of the solutions, and placing the mixture back into each compartment of the cell. The mixing required 3–4 min, after which spectra were taken frequently during the course of the complementation reaction.

In calculating difference spectra, slight path length differences in the two compartments of the sample cell were corrected for on the basis of spectra taken with tryptophan solutions having A_{280} values equivalent to those of the experimental CB2 and M15 protein solutions. Spectra were taken before and after mixing the tryptophan solutions and comparison indicated a path length difference of about 0.004 cm between the compartments.

Results

Conditions of Complementation. The time course of the reaction is shown in Figure 1. The effects of different buffer systems on complementation are summarized in Table I. It can be seen that complementation is very poor in buffers containing bicarbonate. To test for ionic strength effects, sodium phosphate concentration was varied from 0.05 to 0.5 M; no differences were seen over this range. Optimal complementation occurred in buffer A–BSA (pH 7). A gradual decrease in final activity generated, to about 80% of maximal, was observed as the pH was either increased from 7 toward 8, or decreased toward 6.

An additional finding of some interest is that complementation is completely suppressed in the absence of thiol reagents. β-Mercaptoethanol (0.05 M), dithioerythritol (0.04 M), and cysteine (0.1 M) each sustain the complementation. A number of other compounds were substituted for thiol, including chelating agents (EDTA, 10^{-3} M; EGTA, 10^{-3} M; o-phenanthroline, 10^{-3} M, 10^{-4} M), alcohols (methanol, 1 M, 2 M; ethanol, 0.5 M), a substrate analogue (IPTG, 4×10^{-4} M),

TABLE I: Influence of Buffer Ions on Complementation.[a]

Buffer[b]	Excess M15,[c] % of Max Act.	Excess CB2,[d] % of Max Act.
0.1 M Sodium phosphate	100	100
0.02 M Tris-HCl	40	35
0.1 M NH$_4$HCO$_3$	1	1
0.1 M Potassium phosphate	37	90
0.1 M NaHCO$_3$	1	1

[a] Total volumes during complementation were 0.2 ml; incubation time was 3 h. [b] All at pH 7 and containing 0.05 M β-mercaptoethanol, 1 mM MgSO$_4$, 0.2 mM MnSO$_4$, in addition to indicated salts. [c] Excess M15: M15 protein = 10^{-6} M, CB2/M15 = 1/20. [d] Excess CB2: M15 protein = 2×10^{-8} M, CB2/M15 = 75/1.

a reducing agent (NaBH$_4$, 4 mg/ml), and a protein stabilizing agent (poly(ethylene glycol), 1 mg/ml). Of these, only EGTA and EDTA permitted complementation. In control experiments, none of the compounds affected the activity of β-galactosidase when present during a 3-h incubation period, or when included in the β-galactosidase assay buffer.

The optimal concentrations in the reaction mixture were approximately 0.05 M for β-mercaptoethanol and dithioerythritol, and 10^{-3} M for EGTA. A slight stimulatory effect of Mg was also observed (in the presence of β-mercaptoethanol and EGTA), reaching a maximum at about 10^{-3} M. Preincubation (2.5 h) of M15 protein in the absence of thiol markedly reduced the efficiency of complementation to about 20% of maximal, whereas similar preincubation of CB2 had no effect. Inactivation occurs very quickly, since complementation is abolished when M15 protein and CB2 are removed from thiol (by dilution) only at the start of complementation. Complementation of M15 protein with CB2 eliminates the thiol requirement, since both complemented M15 and β-galactosidase are quite stable in thiol absence.

The influence of urea on complementation was also investigated. With increasing urea concentrations from 0 to 1 M, complementation efficiency declined steadily; no complementation occurred at urea concentrations higher than 1 M.

Properties of the Complemented Enzyme. By equilibrium sedimentation in the ultracentrifuge, the complemented enzyme appeared homogeneous, with a molecular weight of 533 000 ± 25 000. This is approximately twice the molecular weight of the M15 dimer and indicates that the complemented enzyme is tetrameric.[2] Gel filtration experiments confirmed this conclusion, since complemented enzyme elutes at the same position as β-galactosidase, while M15 protein elutes later (Figure 2).

The gel filtration study in Figure 2 also demonstrates physical binding between M15 protein and [CM-^{3}H]CB2 in the complemented enzyme. The ^{3}H label provides a marker for CB2, and forms a peak symmetrical with the β-galactosidase activity peak of the active complex. The ratio of CB2/M15 monomer in this peak (fraction A) was 0.7. Because the column was not run under optimal buffer conditions, the ratio serves only as a rough estimate of the stoichiometry of interaction.

[2] The terms "dimer" and "tetramer" used with respect to the complemented enzyme refer to the number of M15 monomers in the complex. Note that, assuming a 1:1 stoichiometry of interaction between CB2 and M15 protein, a molecular weight of 4(132 000) + 4(10 300) = 569 200 would be expected for the active complex.
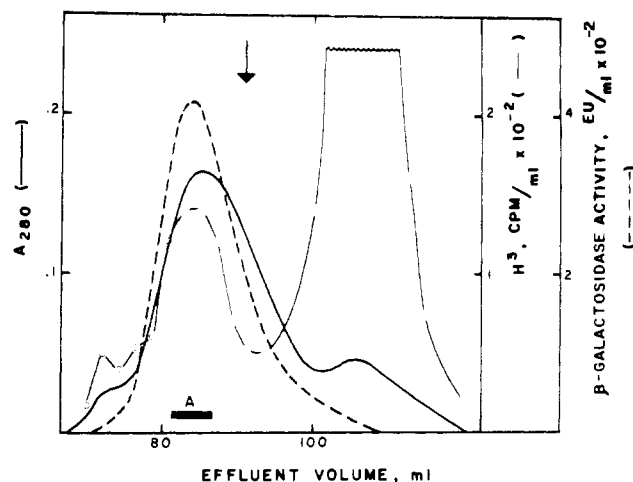

FIGURE 2: Physical binding between M15 protein and CB2. Sixty nmoles of [CM-^{3}H]CB2 (320 cpm/nmol) was combined with 20 of nmol M15 protein in buffer B, 0.005 M β-mercaptoethanol, incubated for 9 h at 28 °C, and applied to a Bio-Gel A-1.5 m column (1.5 × 90 cm) equilibrated at room temperature in the same buffer. Two-milliliter fractions were collected at a flow rate of 30 ml/h. The fractions were monitored by A_{280}, ^{3}H cpm, and β-galactosidase activity, and pooled fraction A was made. The elution position of M15 protein is indicated by the arrow. In a control run with CB2 alone, some aggregated CB2 (about 15% of total) eluted near the void volume. Correction was made for this in calculating the ^{3}H profile shown. In addition, when CB2 was incubated for 9 h with β-galactosidase, rather than M15 protein, and applied to the column, only small amounts of ^{3}H eluted with the β-galactosidase.
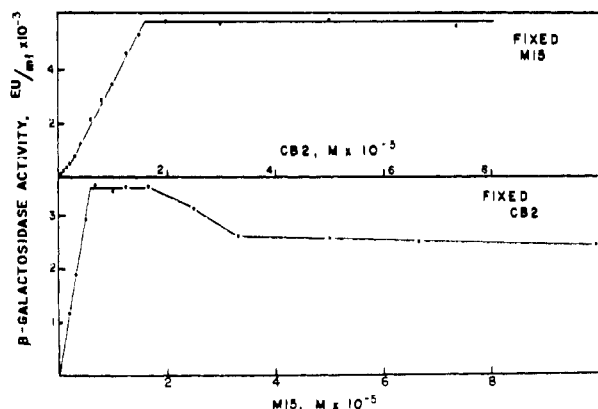

FIGURE 3: Stoichiometry. Fixed concentrations (10^{-5} M) of M15 protein or CB2 were incubated with the indicated range of concentrations of the other component for 9 h and then assayed for β-galactosidase activity.

To more accurately measure the stoichiometry, a fixed concentration of M15 protein (10^{-5} M) was complemented with increasing concentrations of CB2, and vice versa (Figure 3). The concentrations were made high enough so that complementation would go to completion in every case (see below), and the break points in the plots are then indicative of stoichiometry. Both plots give values of 1.6 CB2/1 M15 monomer.

The sigmoidicity at low CB2 concentrations in the fixed M15 protein plot and the declining activity at high M15 protein concentrations in the fixed CB2 plot indicate a type of cooperativity in the CB2-M15 protein interaction—partially complemented M15 protein is less active than expected by proportionality.

For further characterization, partially complemented M15 protein, fully complemented M15 protein, and β-galactosidase
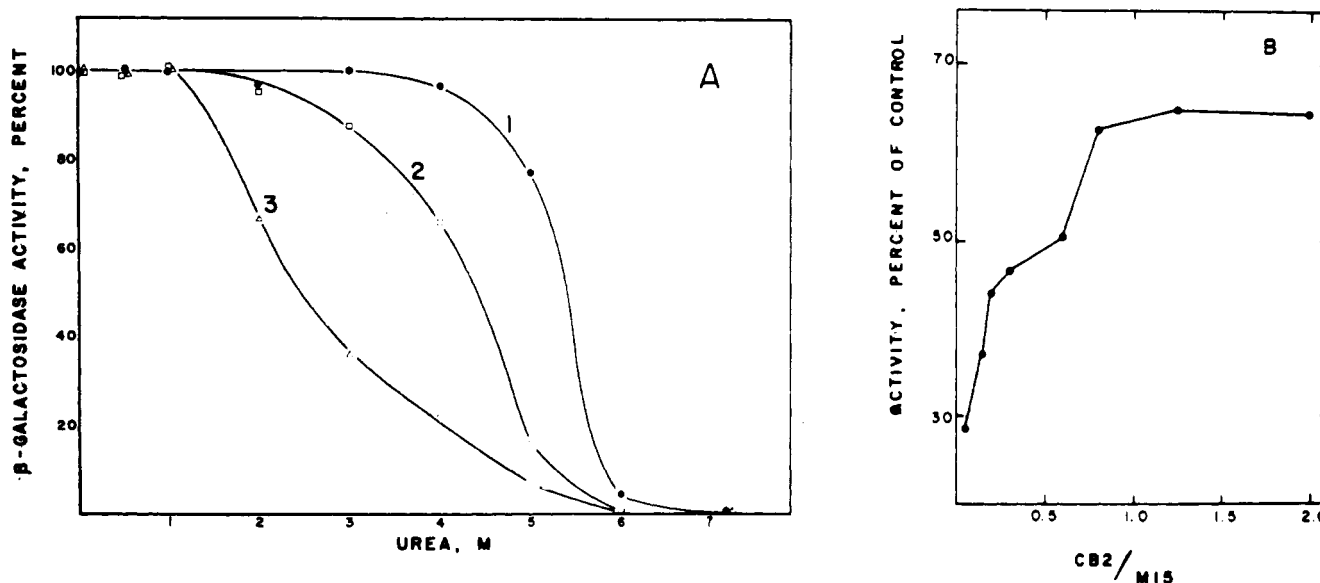
FIGURE 4: (A) Effect of urea on β-galactosidase and on complemented M15 protein preparations. The enzyme preparations were incubated for 2 h at room temperature in buffer A, 0.05 mg/ml of BSA with the indicated urea concentrations and aliquots were diluted into buffer B for β-galactosidase assays. As a control for reactivation after dilution of urea during assays, assays were also performed in buffer A containing the same concentration of urea as present during incubation; similar results were obtained. The data are expressed as per cent of activity remaining, relative to the value for no urea. Curve 1: β-galactosidase, 10^{-7} M; Curve 2: fully complemented M15 protein (CB2/M15 protein = 2/1), 10^{-7} M with respect to M15 protein; Curve 3: Partially complemented M15 protein (CB2/M15 protein = 1/10), 10^{-6} M with respect to M15 protein. (B) Relationship between degree of complentation and stability in urea. Complemented M15 protein preparations with the indicated CB2/M15 protein ratios were incubated in buffer A, 3.2 M urea, and 0.05 mg/ml of BSA for 2 h at room temperature, and in buffer A, 0.05 mg/ml of BSA (controls) for the same period. β-Galactosidase assays were then performed. The extent of inactivation in 3.2 M urea is expressed as the percent activity remaining relative to the respective control. The concentration with respect to M15 protein was 10^{-6} M in every case.

were compared with respect to K_m for substrate (ONPG), susceptibility to urea, an heat inactivation.

For the K_m determinations, activities were measured over an ONPG concentration range of 2×10^{-4}–10^{-3} M and the (fixed) enzyme concentrations were 2×10^{-9} M for β-galactosidase, 2×10^{-9} M for M15 protein in the fully complemented enzyme (CB2/M15 protein = 3/1), and 50×10^{-9} M for M15 protein in the partially complemented enzyme (CB2/M15 protein = 1/20). Data were also taken for the low β-galactosidase activity present in M15 protein preparations (see Methods) with M15 protein at a concentration of 4×10^{-6} M. Data were plotted according to the Lineweaver–Burk and Eadie–Hofstee methods. For each case, the K_m determined from these plots equalled 2.2×10^{-4} M ($\pm 0.2 \times 10^{-4}$ M).

For K_I determinations the same ONPG concentration range was used, with the same enzyme concentrations, and an IPTG concentration of 5×10^{-4} M. The inhibition by IPTG was competitive with ONPG for each enzyme preparation, as expected (Wallenfels and Weil, 1972). K_I values were obtained from the equation

$$K_I = \frac{K_m I}{K_m(\text{apparent}) - K_m}$$

As with K_m, the K_I values were all very close: $K_I = 8 \times 10^{-4}$ M ($\pm 0.7 \times 10^{-4}$ M).

In contrast to K_m and K_I values, clear differences were found in comparing the enzymes with respect to urea susceptibility (Figure 4A). β-Galactosidase is least susceptible and partially complemented enzyme most susceptible. In addition, urea susceptibility is a function of the degree to which M15 protein is complemented by CB2, as shown by the experiment in Figure 4B.

The final comparison between the enzymes was by heat inactivation. One volume of enzyme solution at 25 °C was added

to 9 volumes of buffer A–BSA at 60 °C to give a temperature of approximately 57 °C. Incubation was then carried out at 57 °C for 5 min and aliquots were removed every 0.5 min and assayed for β-galactosidase activity. The concentrations during incubation were β-galactosidase, 10^{-7} M; fully complemented M15 protein (CB2/M15 protein = 3/1), 10^{-7} M with respect to M15 protein; and partially complemented M15 protein (CB2/M15 protein = 1/5), 10^{-6} M with respect to M15 protein. It was found that the partially and fully complemented M15 proteins were more heat labile at 57 °C than β-galactosidase. Respective half-times of inactivation were about 4 and 17 min. No difference was observed between the partially and fully complemented M15 proteins.

Equilibrium Constant for the Complementation Reaction. At an M15 protein concentration of 10^{-8} M and CB2 concentrations of 10^{-7} and 10^{-6} M (for examples), the time course of the complementation reaction differs considerably (Figure 1), but the end points reached are the same and indicate that the reactions go to completion in both cases. Therefore, the concentrations used are in excess of the overall K_d for the complementation, where $K_d = 1/K_{eq}$ and K_{eq} is the overall equilibrium constant. To estimate a value for K_{eq}, complementation was carried out over a concentration range near K_d (Figure 5). Except for the much lower CB2 and M15 protein concentrations, these experiments are equivalent to the stoichiometry experiments in Figure 3. Again, the sigmoid character of the fixed M15 protein plot and the activity decline in the fixed CB2 plot can be seen. As noted in Figure 6, care was taken to be sure that the complementation reactions had reached equilibrium before the β-galactosidase assays were performed. Equivalent results were obtained in experiments with BSA omitted from the reaction mixture.

K_{eq} estimates from the data of Figure 5 were made by the graphical linearization method of Berger and Levit (1973) and
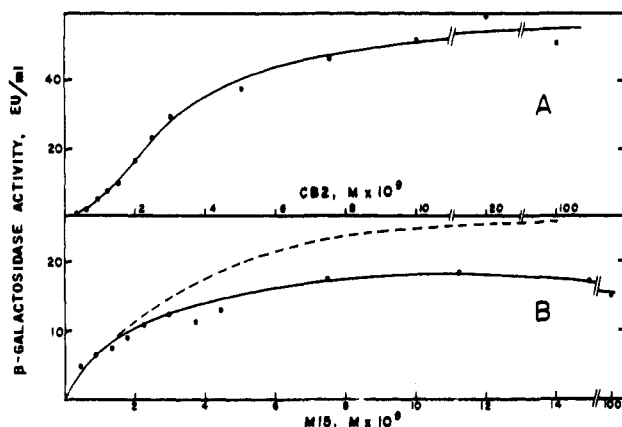
FIGURE 5: Stoichiometry of interaction between CB2 and M15 protein at low concentrations. (A) Tubes with fixed M15 protein concentration of 10^{-9} M and the indicated concentrations of CB2 were prepared. To determine when equilibrium was reached in the complementation reaction, appearance of activity in the tube with lowest CB2 concentration was monitored. After 50 h, all tubes were assayed for activity. (B) Experiment equivalent to A except that fixed CB2 at 10^{-9} M was used with the indicated concentrations of M15 protein. The data have been corrected for the β-galactosidase activity present in the M15 protein preparation. The dashed line estimate represents the data modified, so as to eliminate the decline in activity observed at CB2/M15 protein ratios less than 1. The modifications were done by calculating the concentration of bound CB2 at each point using an assumed K_{eq} of 10^{-9} M. The ratio bound CB2/M15 protein was then calculated and the activity was modified in accordance with Figure 3 (fixed CB2).

also by the graphical method of Richards and Vithayathil (1959). The analyses were complicated by the fact that the saturation plots were not truly hyperbolic, but both procedures gave roughly $1-2 \times 10^9$ M^{-1} for the overall value of K_{eq}.

This rather high K_{eq} is confirmed by another experiment, in which complemented M15 protein (CB2/M15 protein = 2/1; M15 protein = 10^{-5} M) was diluted into buffer A-BSA. For dilutions giving concentrations as low as 10^{-9} M (with respect to M15 protein), no loss in β-galactosidase activity was observed for at least 44 h at 28 °C.

Possible questions concerning the K_{eq} determinations are the extent of M15 protein stability during the 50-h incubation at 28 °C prior to assay, and the effect of substrate (present in the final assays) on the position of the equilibrium. However, no loss in α-acceptor activity of M15 protein (10^{-5} M, or 1.32 mg/ml) was observed over a 48-h incubation at 28 °C in buffer A-BSA, and, to test for substrate effects on the equilibrium, complementation was carried out in the presence of the substrate analogue IPTG at 10^{-2} M. This concentration compares to a level of 2.6×10^{-3} M for ONPG in the β-galactosidase assays. The concentrations of both CB2 and M15 protein were 10^{-6} M, and the appearance of β-galactosidase activity over a 5-h period was monitored by removal and assay of 5-μl aliquots. The assay times throughout were 2 min. A slight increase ($\sim$10%) in the final level of activity reached was observed for the case of complementation in the presence of IPTG, but no effect on the kinetics of the complementation was apparent.

Initial Velocity Studies. To determine the order of the complementation reaction, a series of initial velocity (v_0) studies was performed using a Cary 16 spectrophotometer to facilitate the v_0 measurements. ONPG was included in the reaction mixture and v_0 values, expressed as EU/ml·min, were obtained by plotting the slope of the A_{420} recording as a function of time. v_0's were determined for a fixed M15 protein concentration with excess, increasing concentrations of CB2,

and vice versa (fixed CB2). These experiments are summarized in Figure 6. Figure 6A shows fixed CB2 with M15 protein excesses of $\geq$4.5-fold. In Figure 6B, M15 protein is fixed and the CB2 excess is $\geq$6-fold. In each case, saturation-type curves are obtained. This suggests that the complementation mechanism(s) involves a unimolecular rate-determining step at the higher C_0 concentrations where v_0 levels off; i.e., the reaction(s) becomes zero order with respect to the excess component as the latter's concentration is increased. The most likely possibilities for a unimolecular step are conformational change in (a) M15 protein, (b) CB2, or (c) a (rapidly-formed) complex containing M15 protein and CB2.

Figure 6 also shows transformations of the saturation data. Double-reciprocal plots (Figure 6C) allow estimates of V_m ($1/y$ intercept) and *apparent* K_d ($-$ ($1/x$ intercept)). From V_m, a rate constant (k) for the unimolecular step can be calculated according to the equation

$$V_m = k(C_0)$$

where C_0 = initial concentration of fixed component. V_m must be expressed in M/min rather than EU/ml min, and in making this conversion, a value of 50 000 EU/nmol was used for the activity of complemented enzyme with excess CB2, and 30 000 EU/nmol with excess M15 protein. k and V_m values are summarized in Table II. The apparent K_d values, also shown in Table II, are close to 10^{-6} M for both experiments. It can be noted that this is greater by a factor of approximately 10^3 than the overall K_d value for the complementation reaction (above).

Treatment of the v_0 data in accordance with the Hill equation is shown in Figure 6D. The Hill slope in each case is close to 1.

v_0 measurements were also made at various equimolar concentrations of CB2 and M15 protein. The data, shown in Figure 7A, are again consistent with a saturation type of phenomenon: the curve is sigmoid, but does not appear to level off. One would expect that v_0 would continue to increase linearly at concentrations higher than those shown. In Figure 7B, v_0's for the lower concentrations are plotted as a function of the square of concentration. A roughly linear increase in v_0 is seen and assuming that the rate equation applies

$$v_0 = k_{app}(CB2_0)(M15_0) = k_{app}(C_0)^2$$

then

$$k_{app} = slope = 4.7 \times 10^{15} \frac{\dfrac{EU}{ml\ min}}{M^2} = 9.4 \times 10^4\ M^{-1}\ min^{-1}$$

Uv Difference Spectroscopy During Complementation. As a physical chemical probe for structural changes occurring during complementation, the uv absorbance of the mixed and unmixed components was compared by difference spectroscopy. Buffer C was used to avoid artifacts due to fluctuation in thiol absorbance. Figure 8A shows the calculated difference spectra for early and late times. The experiment was also performed in buffer A, with qualitatively similar results, although a rising baseline obscured the results somewhat and the high thiol absorbance resulted in a higher noise-signal ratio. In a control experiment carried out in buffer A with β-galactosidase in place of M15 protein, no change in the A_{297} was observed.

Figure 8B compares the kinetics of activation to the change in A_{297} for the experiment of Figure 8A. Some correlation between these parameters is evident, but a proportionately
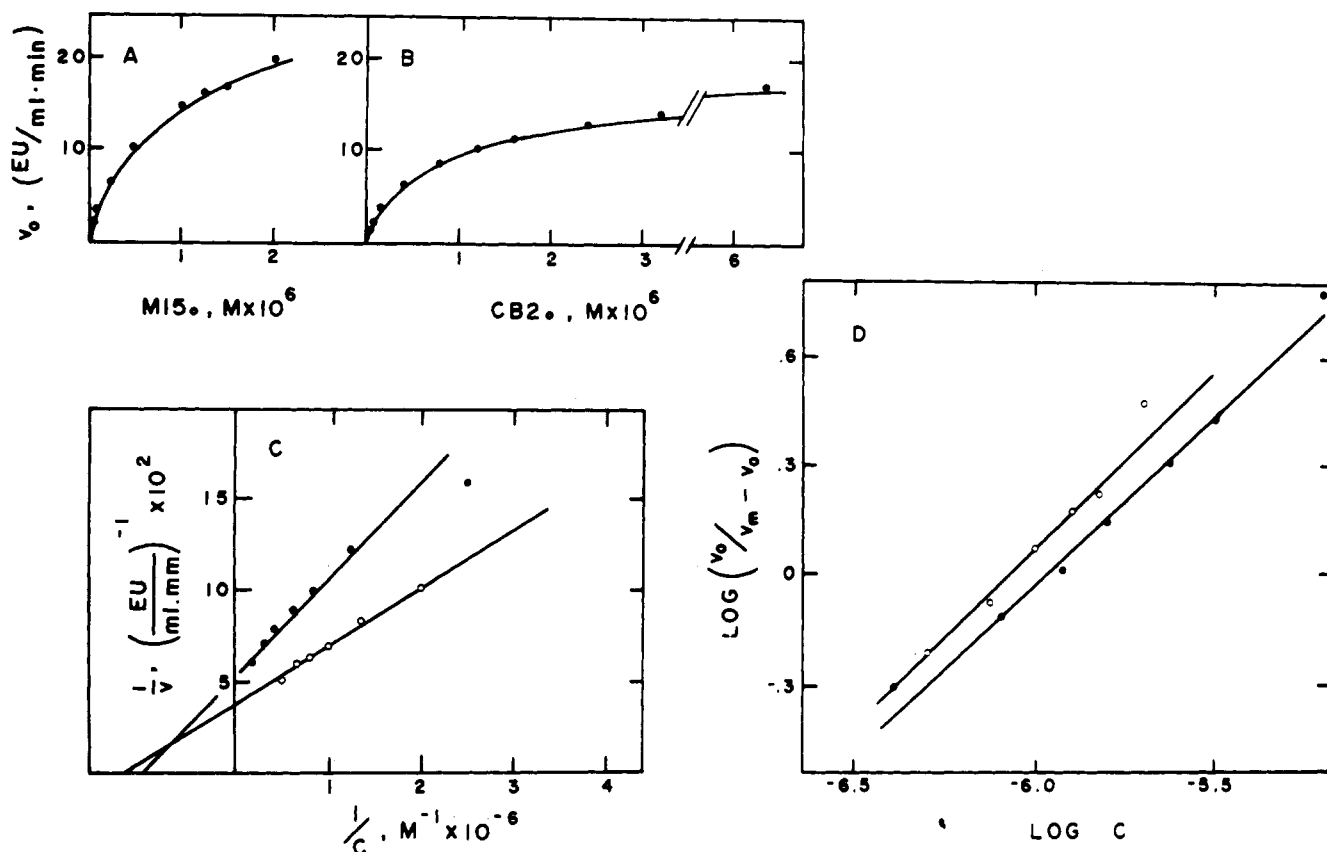
FIGURE 6: Saturation curves for complementation reaction. v_0 measurements for the reaction were made in a Cary 16 spectrophotometer as described under Methods, with a fixed concentration of one component and various excess concentrations of the other component as indicated. (A) Fixed CB2 = 11.2×10^{-9} M. (B) Fixed M15 protein = 7×10^{-9} M. (C) Double-reciprocal plots of the data in A and B, according to the equation $1/v_0 = 1/V_m + ((K_d/V_m)(1/C_0))$. (D) Hill plots of the data in A and B, according to the equation $\log (v_0/(V_m - v_0)) = n(\log C_0) + \log K_a$; n = approximately 1 for both cases. Open circles, fixed CB2; closed circles, fixed M15 protein.
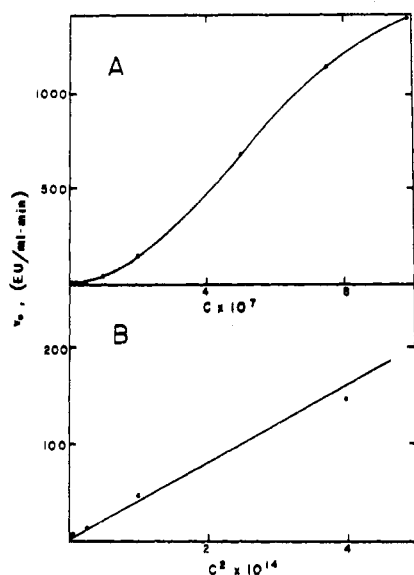

FIGURE 7: Initial rates as a function of M15 protein and CB2 concentrations. v_0 for the complementation reaction was measured in a Cary 16 spectrophotometer (see Methods) with a series of equimolar concentrations of CB2 and M15 protein. (A) v_0 plotted as a function of concentration. (B) v_0 plotted as a function of the squaee of concentration.

larger increase in A_{297} appears to have occurred early in the reaction. If one assumes that the A_{297} change is partially caused by a rapid process (such as CB2 binding), a hypothetical curve representing the kinetics of the remaining change

can be obtained. Such a curve is shown in Figure 8B, with 40% of the change taken as rapid and, therefore, subtracted away. It can be seen that the correlation to the activity curve is improved.

Temperature Dependence of Complementation. In order to estimate the activation energy (E_a) of the unimolecular rate-determining step, the temperature dependence of α complementation was investigated. Arrhenius plots, from v_0 measurements of 0, 14, and 28 °C, were made for complementation with excess M15 protein and with excess CB2. The initial rates were measured by removal and assay of aliquots from the complementation reaction mixture. Assays were performed at the same temperature as the complementation and converted to values for 28 °C on the basis of temperature dependence of β-galactosidase activity in the assay. v_0 was expressed as EU/ml min. Concentrations were 10^{-5} M for the excess component and 10^{-6} M for the limiting component. For both excess CB2 and excess M15, the points on the Arrhenius plots fell very close to a straight line. Also, the slopes of the two lines were similar, indicating similar E_a's. The E_a calculated from the slope was about 24 000 cal.

Discussion

From the work described here, in conjunction with previous studies in this laboratory (Langley et al., 1975a,b), a picture of the molecular nature of α complementation between CB2 and M15 protein is emerging. β-Galactosidase activity can be fully regained as a result of the interaction. The CB2 remains bound to M15 protein, and a stoichiometry of 1.6 CB2 mole-

TABLE II: Kinetic Constants for v_0 Studies of α Complementation.

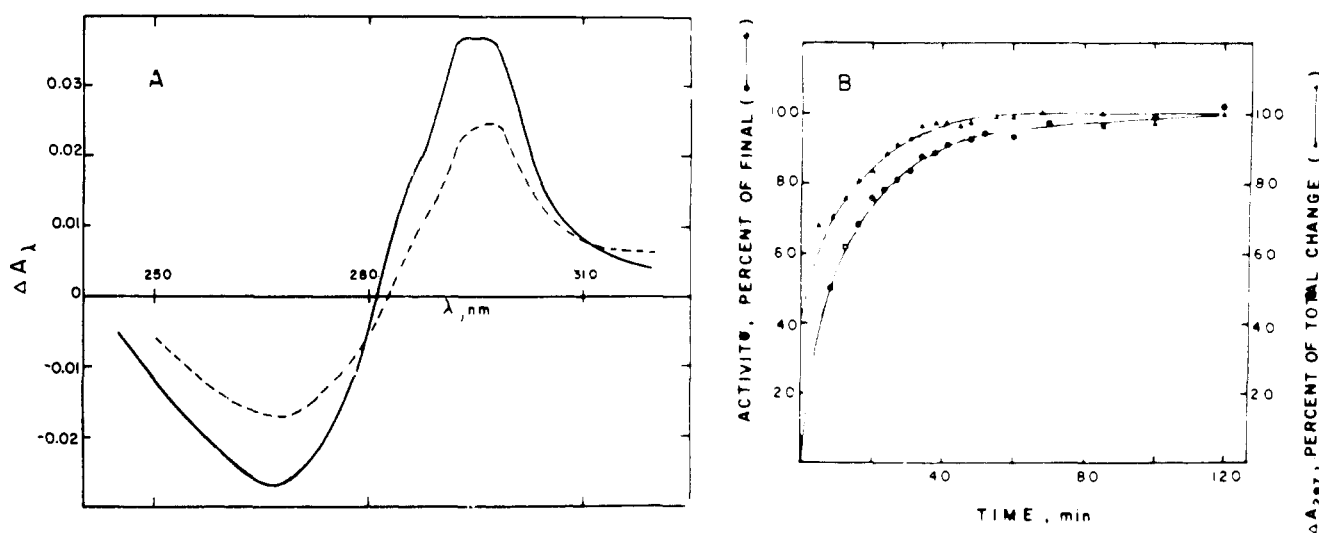| Expt	Fixed Component	C_0 of Fixed Component ($M \times 10^9$)	V_m (EU/ml · min)	V_m (M/min $\times 10^{10}$)	k (min^{-1})	K_d ($M \times 10^6$)
a	CB2	11.2	26.7	8.9	0.12	0.84
b	M15 protein	7.0	19.2	3.8	0.055	1.0

FIGURE 8: (A) Uv difference spectrum. The uv absorbance of M15 protein plus CB2 was compared before and after mixing, by means of difference spectroscopy using the tandem cell technique as described under Methods. Buffer C was used. M15 protein concentration after mixing was 1 mg/ml (7.5×10^{-6} M) and CB2 0.12 mg/ml (12×10^{-6} M). Dashed line, difference spectrum taken approximately 5 min after mixing the components in the sample-beam cuvette. Solid line, difference spectrum taken approximately 2 h after mixing. Appearance of activity was essentially complete after 2 h (see B). (B) Kinetics of appearance of activity and change in A_{297} during complementation. Data are from the experiment illustrated in A. Closed circles, appearance of activity; triangles, ΔA_{297}; open circles, ΔA_{297} with 40% "baseline" subtracted (see text); all expressed as a percent of total change.

cules per M15 protein monomer in the active complex has been determined experimentally. This deviates from the anticipated 1:1 ratio, and we speculate that 1:1 is, in fact, the true value, but that inactive CB2 molecules are present and account for the high ratio obtained. The complementation interaction also restores the ability to form structures that are tetrameric in the sense of containing four functional units.

The apparent cooperativity of interaction in the stoichiometry experiments, where low CB2/M15 protein ratio results in activities lower than expected by proportionality, is an interesting phenomenon. One explanation is that the catalytic sites in M15 complexes not saturated with CB2 are less active than the sites in saturated M15 complexes. If so, the decreased catalytic activity is a consequence of decreased V_m for substrate cleavage, since K_m does not appear to be raised. One can postulate that the uncomplemented M15 monomers in a partially complemented complex could constrain the complemented monomers and prevent full activation. Complementation of more of the monomers in a complex would progressively release any such constraints.

The apparent cooperativity could also be explained on the assumptions that only tetrameric structures are active and that tetramers cannot form if a minimum CB2/M15 protein ratio is not reached. In other words, at low CB2/M15 protein ratios, CB2 might be sequestered on M15 dimers unable to find suitable partners with which to form tetramer. One might expect that sufficiently high M15 protein excess would then cause complementation to be negligible. This does not seem to be the case, but the discussion is complicated by the fact that

if the tetramer formation were an equilibrium reaction, adding more M15 protein would drive it toward completion.

A third possible cause of the apparent cooperativity is that the CB2–M15 protein binding affinity is less when M15 protein is in excess. However, this seems unlikely, since the effect occurs at concentrations much greater than the estimated value of $1/K_{eq}$ for the complementation reaction. Further experiments are needed to distinguish between these possibilities.
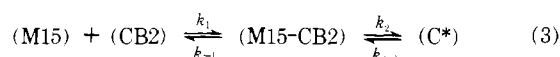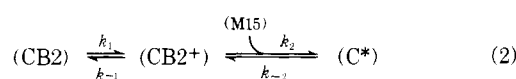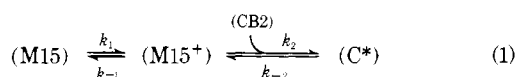
In comparisons of β-galactosidase, fully complemented M15 protein, and partially complemented M15 protein by heat and urea inactivation, the major differences were seen between β-galactosidase and the two complemented enzymes. The kinetics of heat inactivation were similar to those reported by Ullmann et al. (1968) for complementation between fragments produced by mutant strains. Previous work has shown that complemented enzyme takes on all antigenic determinants found in wild-type β-galactosidase (Lin, 1971) and also takes on a resistance to in vivo degradation equivalent to that of β-galactosidase (Villarejo et al., 1972). One can conclude that complemented enzyme mirrors wild-type β-galactosidase with respect to conformational and functional properties, but that differences exist in stability of the conformation and structure when exposed to disruptive conditions, such as heat and urea. The lowered stability of the complemented enzyme probably reflects the fact that the segment provided by CB2 is not covalently linked to the rest of the monomer sequence, as is the case for the wild type enzyme. Instability could also be introduced by the substantial overlap in sequence between CB2 and M15 protein in the active complex (i.e., residues 3–10 and

42-92), and by the absence of residues 1 and 2 of β-galactosidase. Partially complemented enzyme is less stable to urea than fully complemented enzyme. A correlation therefore exists betwenn the increased urea susceptibility of partially complemented enzymes and the fact that their activity is lower than expected by proportionality.

The estimated overall K_{eq} of $1-2 \times 10^9$ M^{-1} for the complementation reaction indicates that the process is virtually irreversible. For complementation between ribonuclease S peptide and S protein, the presence of substrate increases the K_{eq} by a factor of 10^4 (Woodfin and Massey, 1968). We tested for equivalent effects on α-complementation by adding IPTG, and observed no effect on the kinetics of complementation and only slight effect on the final level of activity reached. Since a substrate analogue had to be used rather than ONPG, and since ONPG was present during the β-galactosidase assays by which complementation was monitored, this test does not completely rule out significant effects of ONPG on the complementation reaction and equilibrium position. However, such effects seem unlikely because they would have to occur rapidly, while the complementation reaction is relatively much slower.

A priori, knowing the physical states of CB2, M15 protein, and the active complex, one can suggest several mechanistic possibilities for the complementation reaction. Presumably, a change in conformation of CB2 and/or M15 protein must occur, but this could come about before or after productive interaction between the two. Inactive forms of M15 protein (or CB2) could be in equilibrium with an "active" conformation and reaction with the other component would pull this equilibrium toward the active form. Alternatively, bimolecular complex formation between inactive forms could take place first, followed by conformational change and activation. For any of these possibilities, the order of the reaction would of course depend on which step was rate determining. Tetramer formation could be imagined to occur at any step along a proposed pathway.

From the saturation-type curves in initial velocity studies, it is clear for both fixed M15 protein and for fixed CB2 that the rate-determining step is unimolecular at sufficiently high concentrations of the excess component. The following three reactions have been considered in attempting to explain the saturation type data:[3]

$$(M15) \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} (M15^+) \underset{k_{-2}}{\overset{\overset{(CB2)}{k_2}}{\rightleftarrows}} (C^*) \qquad (1)$$

$$(CB2) \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} (CB2^+) \underset{k_{-2}}{\overset{\overset{(M15)}{k_2}}{\rightleftarrows}} (C^*) \qquad (2)$$

$$(M15) + (CB2) \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} (M15-CB2) \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} (C^*) \qquad (3)$$

where $(M15^+)$ = activated or activatable form of M15 protein; $(CB2^+)$ = activated or activatable form of CB2; (C^*) = active complex between M15 protein and CB2.

Reaction 1 or 3 (but not 2) can explain the saturation-type data with fixed M15 protein, while reaction 2 or 3 (but not 1) can explain the data with fixed CB2 (see Appendix). Only

reaction 3 can explain both sets of data; for explanations other than reaction 3, one must propose different rate-determining steps for the two situations. Consequently, we favor reaction 3 as the mechanism for α complementation. Further support for reaction 3 is the fact that the first-order rate constants (Table II) estimated from V_m for fixed CB2 and fixed M15 protein are quite similar. According to reaction 3, these would represent k_2.

At less than saturating concentrations, the rate constant could be first or second order, depending on which reactions, 1-3, one considers. Reactions 1 and 2 would both imply that the saturation-type curves are the result of a change in the rate-determining step as concentration of the excess component is increased; i.e., at low concentration, the rate-determining step would be bimolecular and, therefore, concentration dependent. For reaction 3, the saturation-type curves could reflect a similar change in the rate-determining step, or else the position of an equilibrium, occurring prior to the rate-determining step, involving complex formation from CB2 and M15 protein, where $K_a = (M15-CB2)/(M15)(CB2)$. As indicated in the Appendix, one cannot distinguish between these various possibilities on the basis of saturation kinetics.

In the v_0 studies with equimolar concentrations of CB2 and M15 protein, the data can be explained by any one of the reactions, 1-3, since they all indicate dependence of v_0 on C_0^2 at low C_0 values, and on C_0 at high C_0 values. In addition, at the lower concentrations, k_{app} in these experiments could be first or second order. Using the equation

$$v_0 = k_{app}(CB2_0)(M15_0) = k_{app}C_0^2$$

k_{app} of 9.4×10^4 M^{-1} min^{-1} was calculated from the data in Figure 8B. If the complementation reaction is, in fact, bimolecular over this concentration range, then k_{app} stands as a second-order rate constant. On the other hand, according to a model of rapid complex formation between M15 protein and CB2 followed by rate-determining conformational change within the complex,

$$k_{app} = kK_a$$

where K_a equals the equilibrium constant for complex formation, and assuming that the concentration of complex is much less than C_0. Using $K_a = 1/K_d = 1.1 \times 10^6$ M^{-1},

$$k = \frac{k_{app}}{K_a} = 0.085 \text{ min}^{-1}$$

The fact that this value is again fairly close to the k derived from V_m of the saturation curves (Table II) is consistent with a rapid complex formation mechanism for complementation.

From the various v_0 studies, then, we favor reaction 3 as the mechanism for complementation, but reactions 1 and 2 are not ruled out (for the cases where they could apply).

Reaction 3, as well as 1 and 2, involves multiple steps. The great difference between the estimated overall K_{eq} and the apparent K_a from the saturation curves is interesting in this regard. For example, in terms of the rapid complex formation model, K_a could correspond to the equilibrium constant for complex formation (if $k_2 \ll k_{-1}$). Subsequent steps, such as conformational change and tetramer formation, would then have individual K_{eq}, which, when multiplied by the apparent K_a for the first step, would give the overall K_{eq} of about $1-2 \times 10^9$ M^{-1}.

The uv difference studies of complementation are consistent with the complex formation mechanism which we propose. A difference peak in the 290-300 nm region appearing during

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[3] The process of tetramer formation is not considered. It could occur at any stage along the reactions written. We assume here that it is not rate determining. It is also recognized that any two, or all three, of the reactions written could be combined into one sequence. For purposes of clarity, they are considered separately. The derivations of the rate equations are given under Appendix.

the interaction is clearly indicated. The interpretation of this difference, by comparison to studies of Yanari and Bovey (1960) with proteins and model systems, is an increase in the hydrophobicity of the environment of tryptophan residues in CB2 and/or M15 protein. Such an increase seems quite plausible, as interactions with amino acid side chains could replace interactions with solvent upon the association of CB2 with M15 protein, or upon the dimer to tetramer transition. It is likely that the difference peak represents the sum of several types of interaction involving several different tryptophan residues, and the kinetics of increase in the peak relative to the kinetics of activity appearance bears this out. There appears to be a rapid increase in $A_{290-300}$ followed by a slow increase which parallels the activity increase quite closely. One can suggest that the rapid increase is due to binding of CB2 to M15 protein (and/or tetramer formation), and the slower increase to a subsequent conformational change. It is noteworthy that Richards and Logue (1962) detected a rapid uv absorbancy increase, followed by a slower increase, in studies of complementation between ribonuclease S peptide and S protein. They could not correlate the changes to kinetics of activation, however.

From the temperature dependence of complementation, at concentrations where the reaction is unimolecular, we have derived an apparent E_a of approximately 24 000 cal for the rate-determining step. The value is essentially the same for complementation with M15 protein excess and CB2 excess. Although somewhat low compared to the usual range for protein denaturation, the value is consistent with the rate-determining step being a conformational change. For example, Sundaram and Fincham (1964) obtained an apparent E_a of 26 000 cal for the first-order activation of a mutant glutamate dehydrogenase by EDTA, interpreted by them as a conformational change process.

The potentials for studying the α-complementation mechanism are somewhat unique because of the relatively slow time course. In other complementation systems, it has been difficult to correlate observed structural changes to activation, due to the rapidity of reaction. For example, Light et al. (1974) reported that the change in intrinsic fluorescence during complementation between fragments 6–48 and 49–149 of staphylococcal nuclease has a unimolecular rate-determining step. The first-order rate constant was 0.03–0.05 s^{-1}. However, no definite assignment of the species undergoing the rate-determining step could be made from the kinetics. And no correlation could be made between the kinetics of fluorescence change and kinetics of activation. For another system, activation of a *lac* Z mutant protein by anti-β-galactosidase antibody, Celada et al. (1970) have suggested a mechanism very much like the model which we favor for α complementation.

Although we propose that a conformational change is involved as the rate-determining step in α complementation, the possibility that CB2 actually comprises part of the catalytic site in the active complex has not been ruled out by our studies. However, such a role for CB2 appears unlikely, since M15 protein itself can be substantially activated by interaction with antibodies against wild-type β-galactosidase.[4]

Acknowledgments

Appendix

The rate equations for reactions 1–3 (above), derived by steady-state assumptions, are for reaction 1:

$$\frac{d(C^*)}{dt} = k_2(CB2)(M15^+) \qquad (1a)$$

Assuming steady state,

$$\frac{d(M15^+)}{dt} = k_1(M15) - k_{-1}(M15^+)$$
$$- k_2(M15^+)(CB2) = 0$$

Also

$$(M15^T) = (M15) + (M15^+) + (C^*)$$

Solving simultaneously for $(M15^+)$,

$$(M15^+) = \frac{k_1[(M15^T) - (C^*)]}{[k_{-1} + k_2(CB2)] + k_1}$$

Substituting into 1a, rearranging, and assuming $(C^*) \sim 0$ for v_0,

$$v_0 = \frac{k_1(CB2)(M15^T)}{\dfrac{k_1 + k_{-1}}{k_2} + (CB2)}$$

For reaction 2, by reasoning analogous to that for reaction 1,

$$v_0 = \frac{k_1(M15)(CB2^T)}{\dfrac{k_1 + k_{-1}}{k_2} + (M15)}$$

For reaction 3, if $(M15) \gg (CB2)$,

$$\frac{d(C^*)}{dt} = k_2(M15-CB2) \qquad (1b)$$

And since

$$(CB2^T) = (CB2) + (M15-CB2) + (C^*)$$

and, assuming steady state,

$$\frac{d(M15-CB2)}{dt} = k_1(CB2)(M15)$$
$$- (k_{-1} + k_2)(M15-CB2) = 0$$

we have (solving simultaneously)

$$(M15-CB2) = \frac{k_1(M15)[(CB2^T) - (C^*)]}{(k_{-1} + k_2) + k_1(M15)}$$

Substituting into 1b, rearranging, and assuming $(C^*) \sim 0$ for v_0,

$$v_0 = \frac{k_2(M15)(CB2^T)}{\dfrac{k_{-1} + k_2}{k_1} + (M15)}$$

If $(CB2) \gg (M15)$, one obtains (in an analogous manner)

$$v_0 = \frac{k_2(CB2)(M15^T)}{\dfrac{k_{-1} + k_2}{k_1} + (CB2)}$$

Note that if $k_2 \ll k_{-1}$, the quantity $(k_{-1} + k_2)/k_1$ becomes k_{-1}/k_1, which equals K_d for the step M15 + CB2 $\rightleftharpoons$ M15–

CB2; reaction 3 then involves a rapid equilibrium prior to the rate-determining step.

For the three reactions described, the species undergoing the unimolecular rate-determining step at high concentrations of the excess component would be (1) M15 protein (dimer), (2) CB2, or (3) a CB2–M15 protein complex. (Reaction 3 closely resembles the Michaelis–Menten formulation of enzyme catalysis.) More specifically, relating the rate equations to the saturation-type curves, one finds, for the fixed CB2 situation, that reaction 2 could apply, with the equation reducing to

$$v_0 = k_{obsd}(M15^T)(CB2^T)$$

at the low M15 concentrations, where

$$(M15) \sim (M15^T) \ll \frac{k_1 + k_{-1}}{k_2}$$

or reducing to

$$v_0 = k_1(CB2^T)$$

at the high M15 concentrations, where

$$(M15) \gg \frac{k_1 + k_{-1}}{k_2}$$

or, alternatively, reaction 3 could apply, with

$$v_0 = k_{obsd}(M15^T)(CB2^T)$$

if

$$(M15) \sim (M15^T) \ll \frac{k_{-1} + k_2}{k_1}$$

for the low M15 concentrations. For the high M15 concentrations,

$$v_0 = k_2(CB2^T)$$

if

$$(M15) \gg \frac{k_{-1} + k_2}{k_1}$$

For the fixed M15 saturation-type curve, reaction 1 could apply, or reaction 3 could apply. (In both cases, the equations would reduce at the low and high CB2 concentrations in ways entirely parallel to those shown for the fixed CB2 saturation-type curve.)

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