Monomer−Dimer Equilibrium of Uncomplemented M15 β-Galactosidase from *Escherichia coli*

Clare N. Gallagher and Reuben E. Huber*

Division of Biochemistry, Faculty of Science, University of Calgary, Calgary, Alberta, Canada T2N 1N4

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ABSTRACT: A series of gel filtration, native polyacrylamide gel electrophoresis (PAGE) and sucrose density experiments showed that uncomplemented M15 β-galactosidase is in a monomer−dimer equilibrium and that only under some specific conditions does the equilibrium strongly favor dimerization. The ratio of dimer to monomer increased as a function of the protein concentration, and a very good fit to a theoretical plot of the effect of protein concentration on an associating system of this type was found. The K_{diss} (equilibrium constant for dimer dissociation) was 2.5×10^{-7} M. The addition of 20 mM Mg^{2+} lowered the K_{diss} to 1.5×10^{-7} M, and the addition of 150 mM NaCl lowered the value to 0.4×10^{-7} M. Thiol reagents (2-mercaptoethanol and dithiothreitol) caused the equilibrium to shift totally to the dimeric form. The monomer−dimer equilibrium was also found to be dependent upon the pH. The dissociation increased as the pH was raised to 8.5, but there was a reversal of the equilibrium in favor of dimer formation at pH 9.0. This suggests that one (or more) residues with a pK_a value of about 8.0 is involved. Tyr and Lys were eliminated as possible residues involved and it is, therefore, likely that one or more Cys are involved. Further evidence that uncomplemented M15 β-galactosidase is in a monomer−dimer equilibrium was that the gel-filtration peaks were not totally resolved and that native PAGE bands were diffuse under all conditions except at high thiol concentration.

β-Galactosidase (EC 3.2.1.23) from *Escherichia coli* is tetrameric. Ullman et al. (1967) found that extracts of a mutant *E. coli* with a deletion in the operator-proximal region (α-segment−the region that codes for the N-terminal end) of the *lacZ* gene produced inactive β-galactosidase. Extracts from strains of *E. coli* with point mutations outside of this segment restored the activity. The restoration of activity was probably due to protein complementation. Protein complementation is a process in which a peptide restores activity to a protein with a deletion. The peptide is usually small and inclusive of the sequence deleted. CNBr digestion of wild-type β-galactosidase yielded a fragment that also brought about complementation (Lin et al., 1970) of a mutant β-galactosidase. The peptide was made up of residues 3−92 of the wild-type β-galactosidase (Langley et al., 1975a). The complementation of β-galactosidase (called α-complementation) is usually done with M15 β-galactosidase (Langley & Zabin, 1976). M15 β-galactosidase has a deletion of residues 11−41 (Langley et al., 1975b), and the peptide that brings about complementation includes the deleted residues of M15 β-galactosidase. Some smaller peptides that include the deleted residues also complement. Jacobson et al. (1994) solved the structure of wild-type β-galactosidase. The dimer−dimer interfaces include four-helix bundles with two helices donated by each dimer. The first 50 residues at the N-terminal end are also at the dimer−dimer interfaces and contribute to tetramerization (and subsequent restoration of activity) both by direct contact and by stabilizing the four-helix bundle. Many of these 50 residues are deleted in M15 β-galactosidase and the added α-peptide probably complements by restoring the interactions lost because of the deletion. The structure (Jacobson et al., 1994) also includes a low-affinity Mg^{2+} "site" in the α-peptide region.

The quaternary structure of uncomplemented M15 β-galactosidase is dimeric at high protein, ionic strength, and mercaptoethanol concentrations (Langley et al., 1975b). Preliminary gel-filtration studies in our laboratory showed, however, that uncomplemented M15 β-galactosidase was present both as a monomer and as a dimer at low protein concentrations and in the absence of Mg^{2+}, NaCl, and thiol. Detailed studies were therefore undertaken to determine the actual quaternary structure of the system and to determine how various conditions affect the monomer−dimer ratio. This study is important for several reasons. The M15 β-galactosidase α-complementing reaction is used extensively in molecular biology for blue/white screening. The chromosomal DNA in the *E. coli* cells used for blue/white screening has the gene for M15 β-galactosidase and the plasmids employed have a gene segment that codes for the α-peptide and contain a "multicloning" site. If foreign DNA is not inserted into the multicloning site, normal α-peptide is expressed by the plasmid and the M15 β-galactosidase becomes complemented and is active. If foreign DNA is inserted, expression of the α-peptide is altered and the M15 β-galactosidase is not complemented. The colonies are blue on plates with X-gal[1] if there is no insertion but white if there is insertion. The interactions of M15 β-galactosidase are also models for protein−protein interactions. The system has also found important application in the medical diagnostics field (Engel & Khanna, 1992). Specific antibodies

* Corresponding author: FAX (403) 289-9311; e-mail huber@acs.ucalgary.ca.

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[1] Abbreviations: DTT, dithiothreitol; FPLC, fast protein liquid chromatography; ONPG, *o*-nitrophenyl β-D-galactopyranoside; PAGE, polyacrylamide gel electrophoresis; PNPG, *p*-nitrophenyl β-D-galactopyranoside; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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to α-peptides with specific ligands that are attached chemically are made. α-Complementation does not occur if such an antibody is present in the mixture because the α-peptide with the specific ligand is complexed to the antibody. Concentrations of specific ligands in physiological fluids can then be quantitated by the amount of complementation that occurs as a result of the specific ligands displacing the α-peptides.

MATERIALS AND METHODS

Biochemical Reagents. M15 β-galactosidase was a gift from Boehringer-Mannheim. The chemicals used were purchased from Sigma, ICN, Pharmacia, Bio-Rad, Fisher, or similar sources.

Protein Concentration. The concentration of protein was determined by absorbance at 280 nm. An extinction coefficient of 2.06 absorbance units mg^{-1} mL^{-1} (Wallenfels & Weil, 1972) was used.

Polyacrylamide Gel Electrophoresis. Native electrophoretic analyses were carried out using the PhastGel system (Pharmacia). Except for studies with pH, samples were initially eluted into the running buffer (0.2 M Tris/0.2 M Tricine, pH 8.1) with various additions of reagents (Mg^{2+}, 20 mM; NaCl, 150 mM; 2-mercaptoethanol, 100 mM; and/or DTT, 20 mM) by the use of a FPLC Superose-6 column (FPLC, Pharmacia). The samples were then diluted as necessary using running buffer with the appropriate additions. To determine the effects of pH, the running buffer was adjusted to the desired pH values at 4 °C and the gels were run at 4 °C. The samples were incubated overnight in each case (at 4 °C) before loading and separation to allow the protein to reach equilibrium. The proteins were loaded directly onto native 4−15% gradient gels and electrophoresed for 360 Vh at 4 °C. The gels were stained using Coomassie Blue as described by the manufacturer (Pharmacia). A LKB Bromma ultrascan II enhanced laser densitometer was used to determine the densities of the gel electrophoresis bands.

Gel Filtration. To maintain consistency, the PAGE running buffer (0.2 M Tris/0.2 M Tricine, pH 8.1) was also used for gel filtration. The effects of protein concentration, 20 mM Mg^{2+}, 150 mM NaCl, 100 mM 2-mercaptoethanol (or 20 mM DTT), and pH were determined. The stock protein was again first eluted into running buffer using the Superose-6 gel-filtration column. The samples were incubated overnight at 4 °C under the conditions being investigated and most gel-filtration analyses were done by elution through a Superose-12 column linked in tandem with a Superose-6 column. Elution was in the same buffer as the sample except when 100 mM 2-mercaptoethanol was present. The very high absorbance at 280 nm resulting from the 2-mercaptoethanol masked the protein absorbance. Therefore, the sample was incubated with 2-mercaptoethanol, but the elution was carried out without 2-mercaptoethanol. Buffers were degassed and filtered through 0.22-μm filters (Millipore). Elution was at a flow rate of 0.2 mL/min. The protein was detected by absorbance at 280 nm.

PeakFit, a software program (Jandel Scientific) designed to resolve overlapping peaks, was used to analyze the gel elution profiles. Several peak resolving methods could be selected. The Gaussian method worked the best.

Sucrose Density Gradient Centrifugation. Buffers for sucrose density gradients were the same as used with native PAGE and gel filtration. Gradients of 5−20% with final volumes of 12 mL were set up in polyallomer tubes. Samples were gently layered on top. Centrifugation was with a Sorvall RC70 ultracentrifuge using a Sorvall TH641 swinging bucket rotor (76800*g*, 20 h, 4 °C). An ISCO density gradient fractionator was used to remove and analyze the samples.

Assays. Samples of the M15 β-galactosidase that had eluted through the gel-filtration columns were diluted into 20 mM TES buffer containing 145 mM NaCl, 20 mM $MgSO_4$, and 100 mM 2-mercaptoethanol (pH 7.0). α-Peptide [1:10 (w/w) peptide: protein] was added. After incubation overnight at 25 °C, the assay was initiated by adding 50 μL of the incubated (complemented) enzyme to 2 mM ONPG in buffer without 2-mercaptoethanol. Absorbance was monitored at 420 nm (25 °C). Gel filtration of the complemented enzyme was also studied without Mg^{2+}, without 2-mercaptoethanol, and without NaCl (in 0.2 M Tris/0.2 M Tricine buffer, pH 8.1). Also, a native PAGE of the complemented enzyme was done with 0.2 M Tris/0.2/M Tricine (pH 8.1) without additions of Mg^{2+}, 2-mercaptoethanol, or NaCl.

Difference Spectral Titration. Identical 1-mL samples of protein (1.5 mg/mL) were placed into reference and sample cuvettes of a spectrophotometer at pH 7.0. A difference spectrum (260−310 nm) at high sensitivity (0.01 absorbance unit, full scale) was taken at pH 7.0. A flat line with an absorbance difference of 0 was found. Difference spectra at the same sensitivity at a series of increasing sample pH values (at increments of about 0.25 unit up to pH 10.75) were taken after careful addition of small measured aliquots of 1 M NaOH to the sample cuvette and exactly the same volumes of water to the reference cuvette.

RESULTS

Establishment of Equilibrium. Preliminary analyses showed that the equilibria between monomer and dimer were established in less than 8 h under each condition. However, to be certain that equilibrium was achieved, samples were incubated overnight for periods greater than 12 h.

Effect of Protein Concentration. Three representative FPLC elution profiles at different total concentrations of M15 β-galactosidase are shown in Figure 1. The total amounts eluted (areas under the elution peaks; note that different absorbance scales are used) were proportional to the amounts of protein applied. The dimer peak eluted at approximately 26 mL and the monomer at approximately 28 mL. Each peak eluted within a volume of about 2−3 mL. The proportion of dimer (determined by resolution with the PeakFit program) decreased as the overall protein concentration was lowered. A summary of the PeakFit analyses is presented in Table 1. The data were analyzed (Figure 2) according to the method of Manning et al. (1996). This is a treatment that mathematically relates the protein concentration (in terms of the theoretical maximum concentration of dimer) to the expected amounts of dimer and monomer for an associating−dissociating equilibrium. The slope of the graph obtained should be 1.0 and one can obtain a K_{diss} value from such a plot. A very good fit (Figure 2) to a slope of 1.0 was indeed obtained and the K_{diss} value was calculated to be 2.5×10^{-7} M. The activities (after α-complementation) of samples collected across both peaks (data not shown) were proportional to the protein concentrations.

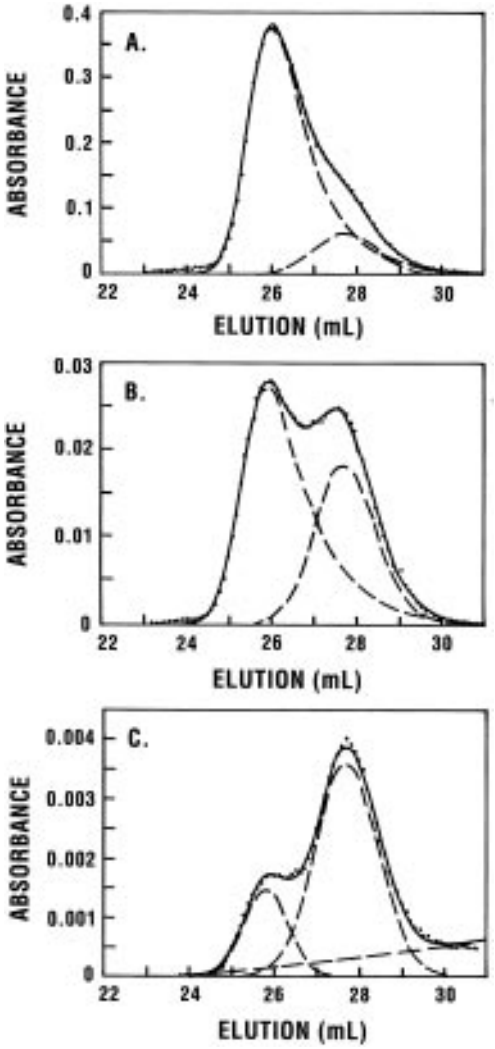

FIGURE 1: Elution profiles of M15 β-galactosidase through a Superose-12 column attached in tandem to a Superose-6 column at three representative M15 β-galactosidase concentrations. The protein was dissolved in 0.2 M Tris/0.2 M Tricine (pH 8.1, degassed and filtered through 0.22-μm filters) and incubated overnight to allow equilibrium to be established. Samples (300 μL) were loaded and eluted in the 0.2 M Tris/0.2 M Tricine buffer and the elution was followed by absorption at 280 nm. (A) 0.5 mg/mL; (B) 0.05 mg/mL; (C) 0.00625 mg/mL. The elution profiles were analyzed by PeakFit to resolve the two peaks. The points indicate the actual absorbencies obtained while the solid line corresponds to the addition of the absorbencies of the monomer and dimer peaks. The fit of the solid line to the points is, therefore, a measure of the fit of the resolved peaks to the data. The dashed lines (− − −) correspond to the monomer and dimer peaks that were resolved by the Peakfit program. In some cases a dashed line also indicates the baseline fit by the program..

The trend of native PAGE results (Figure 3A and Table 2; note that the bands are quite diffuse) was in general agreement with the data from the gel-filtration column (Table 1). However, the data were different and they also did not fit well to a slope of 1.0 when plotted (not shown) by the method of Manning et al. (1996). There could be different staining interactions between the different quaternary forms of the protein, and the gel-filtration results with precise absorbance measurements at 280 nm are probably more reliable.

Effect of Mg^{2+}. Gel filtration (data not shown) showed that if the protein concentration is unchanged, the amount of dimer with Mg^{2+} present was increased relative to the amount without Mg^{2+}. The effect of Mg^{2+} was confirmed with native PAGE. When the running buffer did not contain any Mg^{2+}, the protein ran as a mixture of monomer and dimer (Figure 3A). However, when 20 mM Mg^{2+} was added to the buffer, there was significantly less monomer present (data not shown). Aggregated M15 β-galactosidase was also seen in the presence of Mg^{2+}. Sucrose density centrifugation (not shown) also showed that Mg^{2+} caused a shift in the equilibrium. The protein shoulder was more pronounced in the absence of Mg^{2+} (with EDTA) than in the presence of 20 mM Mg^{2+}. The K_{diss} in the presence of 20 mM Mg^{2+} was calculated to be 1.5×10^{-7} M.

Effect of NaCl. The addition of 150 mM NaCl (at a constant protein concentration) also caused the amount of dimer to increase relative to monomer (gel-filtration data not shown). The K_{diss} in the presence of 150 mM NaCl was low (0.4×10^{-7} M). The increase in the proportion of dimer present upon addition of NaCl was also shown by native PAGE (not shown). There was only a very small amount of monomer present. There was no aggregate band present as was noticed with Mg^{2+}. The bands were quite diffuse. Sucrose density centrifugation with 150 mM NaCl gave a much sharper protein peak than without NaCl (not shown) and no monomer shoulder.

Effect of 2-Mercaptoethanol/Dithiothreitol. A definite shift in the monomer−dimer equilibrium was noted by gel filtration in the presence of 2-mercaptoethanol (not shown)

Table 1: Effect of Concentration on the Percentage of Monomer and Dimer Present with M15 β-Galactosidase during Gel-Filtration Chromatography[a]

concentration (mg/mL)	% dimer	% monomer
0.5	86	14
0.25	74	26
0.1	66	34
0.05	63	37
0.025	41	59
0.0125	36	64
0.00625	22	78

[a] The elution was followed by the absorbance at 280 nm and the amounts of protein present as monomer and dimer were determined using the PeakFit program.

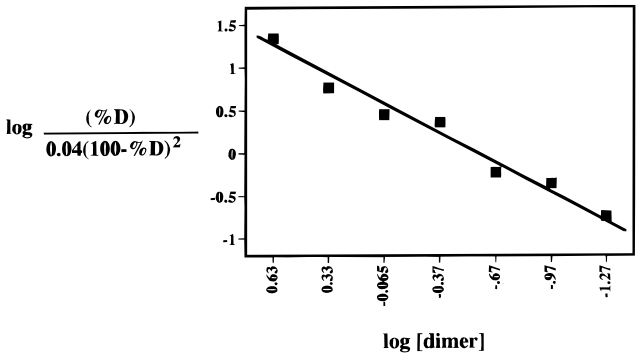

FIGURE 2: Plot of log [dimer] vs log [(% D)/0.04[100 − (% D)]^2]. Here [dimer] represents the concentration of dimer that would be present if the protein were all dimer. % D is the percent of the protein that is actually dimer at the various [dimer]. Manning et al. (1996) showed that if one has an associating system between monomer and dimer and if one plots log [dimer] vs log [(% D)/0.04[100 − (% D)]^2], a straight line with a slope of 1.0 should result and the log [dimer] value that results in the value of log [(% D)/0.04[100 − (% D)]^2] that is equal to 0, is log K_{diss}. A very good fit of the data to a slope of 1.0 when plotted by this method was noted and a K_{diss} value of 2.5×10^{-7} M was obtained.

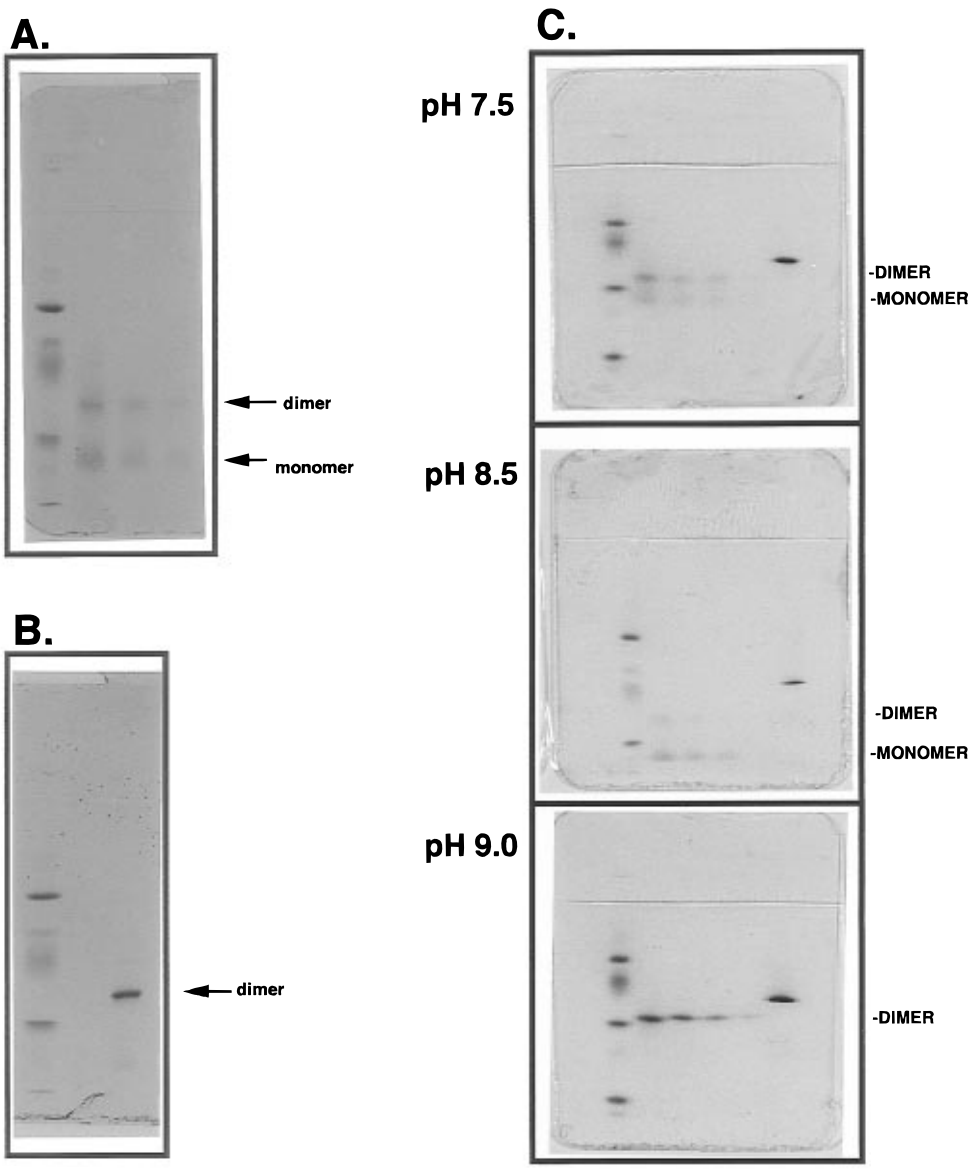

FIGURE 3: Native PAGE of a series of concentrations of M15 β-galactosidase and the effects of various conditions on the native PAGE analysis of monomer and dimer. M15 β-galactosidase was dissolved in 0.2 M Tris/0.2 M Tricine (pH 8.1) and incubated overnight to allow equilibrium to be established. Aliquots were loaded directly onto 4−15% native Phast PAGE gels. After the separations, the gels were stained with Coomassie Blue. (A) Samples without Mg^{2+}. From left: molecular weight standards, 0.5 mg/mL M15 β-galactosidase, 0.25 mg/mL M15 β-galactosidase, 0.1 mg/mL M15 β-galactosidase. (B) Left lane, molecular weight standards; right lane, M15 β-galactosidase (0.5 mg/mL) incubated with 100 mM 2-mercaptoethanol. (C) Native PAGE at three representative pH values. M15 β-Galactosidase was dissolved into 0.2 M Tris/0.2 M Tricine at various pH values. The protein was incubated overnight to allow equilibrium to be established. Aliquots were loaded directly onto 4−15% native Phast PAGE gels. The gels were stained with Coomassie Blue. The rate of migration depended upon the pH (and thus the electrostatic charge of the protein). In each case the left lane is the molecular weight standards and the next four lanes are M15 β-galactosidase samples at 0.5, 0.25, 0.1, and 0.05 mg/mL. The sixth lane is wild-type β-galactosidase (1 mg/mL). The molecular weight standards that were run with each gel were thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; lactate dehydrogenase, 140 kDa; and bovine serum albumin, 67 kDa.

when examined at equal protein concentrations. The protein eluted as a single peak. There was a tailing of the peak, however, and it could be argued that a small amount of monomer was present. Native PAGE, in the presence of both 2-mercaptoethanol (Figure 3B) and DTT (not shown), confirmed the complete shift to dimer. In both cases the dimer band was very sharp and defined and no visible monomer band was present.

All Reagents. Some monomer was present when samples containing Mg^{2+}, NaCl, and 2-mercaptoethanol were eluted through the gel-filtration column system, but there was significantly less than without those reagents (about 90% dimer). This is more than with NaCl or Mg^{2+} alone but lower (unexpectedly) than with only 2-mercaptoethanol. The K_{diss} was calculated to be 0.2×10^{-7} M.

Effect of pH. Samples incubated at varying pH values were analyzed by gel filtration. The results are presented in Table 3. As the pH was increased up to 8.5, the amount of monomer increased. At pH 8.5 there was a large amount of monomer present. Unexpectedly, at pH 9.0 there was mainly dimer present. Figure 3C shows native PAGE gel runs of the equilibria at three representative pH values. The results were very similar to those found by gel filtration. There was more dimer present in the sample than monomer at pH 7.5, but as the pH was raised, the amount of monomer increased. At pH 8.5 there was mostly monomer in the

Table 2: Effect of Concentration on the Percentage of Monomer and Dimer Present in M15 β-Galactosidase As Analyzed by Native PAGE[a]

concentration (mg/mL)	% dimer	% monomer
10	77[a]	12[b]
1.0	87	13
0.5	66	34
0.25	28	72
0.125	21	79

[a] The proportions of monomer and dimer present were determined by the use of an LKB Bromma ultrascan II enhanced laser densitometer. [b] These percentages do not add up to 100% because about 11% of an aggregate form was present at this high protein concentration.

Table 3: Percentage Monomer and Dimer Present As Determined by Gel Filtration in Solutions at Various pH Values[a]

pH	% dimer	% monomer
7.5	61	39
8.0	57	43
8.5	22	78
9.0	100	

[a] The protein concentration in each case was 0.1 mg/mL. Peaks were resolved by PeakFit.

sample but again, at pH 9.0, there was almost only dimer present.

Difference spectra (not shown) at pH values above 7.0 (reference pH was 7.0) showed only minor differences between 260 and 310 nm as a function of pH up to pH 9.0. Between pH 9.0 and 10.25, some definite but very small difference peaks appeared. At pH values of 10.25 and higher, large increases in the difference absorbance spectra were noted. Those large differences indicated that the method used was sensitive enough to detect the ionization of Tyr side chains.

Complemented Enzyme Studies. Gel-filtration experiments (not shown) of the complemented M15 β-galactosidase showed that only tetramer eluted when the runs were done in the Tris/Tricine buffer (pH 8.1) and in the absence of either Mg^{2+} or 2-mercaptoethanol. There was evidence of a small amount of dissociation to dimer when the elution was done without NaCl. No trace of any dimer or monomer was seen (not shown) in a native PAGE experiment of the complemented enzyme in which all of the reagents were left out during the electrophoresis run.

DISCUSSION

Sedimentation equilibrium ultracentrifugation and PAGE experiments have shown that M15 β-galactosidase is a dimer at high protein concentrations and with high ionic strength and high 2-mercaptoethanol concentrations (Langley et al., 1975b). The studies reported here, in which the effects of protein concentration, Mg^{2+}, NaCl, thiol reagents, and pH on the subunit interactions were investigated, clearly show that uncomplemented M15 β-galactosidase exists as a monomer−dimer equilibrium under some conditions. Only under some defined conditions is the equilibrium such that mainly dimer is present.

If a dimer reversibly dissociates, increases in the overall protein concentration should result in more dimer being present. The analyses of various concentrations of M15 β-galactosidase with a gel-filtration column and the plot of the results by the method of Manning et al. (1996) agreed well with this suggestion. Native PAGE analyses results were also in agreement.

Theoretically, if an associating interaction between monomer and dimer is very slow or if no interaction occurs, two distinct peaks should elute, whereas if there is very rapid interaction, only a single intermediate peak should be seen (Ackers & Thompson, 1965; Zimmerman & Ackers, 1970; Uversky, 1993). The gel-filtration peaks were not totally resolved and there may thus have been some interactions occurring during the experiment. However, the peaks eluted at the expected positions relative to molecular weight markers (data not shown). Thus, there was not a large amount of interaction. The bands on the native PAGE were somewhat diffuse (except in the presence of thiols). This shows that a small amount of interaction was also happening during the native PAGE separation.

Some dilution occurs as proteins are eluted through gel-filtration columns. If association−dissociation is rapid, one would expect that the K_{diss} values obtained would be in error because the final protein concentration would be less than the original and the equilibrium should shift. However, the peaks eluted at positions predicted for monomer and dimer and this indicates that any association−dissociation reactions that occurred were slow enough so that the K_{diss} values obtained are quite accurate.

The gel-filtration, native PAGE, and sucrose density centrifugation experiments showed that Mg^{2+} caused the equilibrium between monomer and dimer to shift toward the dimeric form. The K_{diss} value in the presence of Mg^{2+} was 1.5×10^{-7} M (compared to 2.5×10^{-7} M in its absence). The effect of Mg^{2+} on the interaction is probably general, as it is unlikely to be due to specific binding at either of the two Mg^{2+} sites of β-galactosidase. There is a specific high-affinity Mg^{2+} binding site as part of the active site of β-galactosidase (Jacobson et al., 1994) but the amount of Mg^{2+} needed (20 mM) to drive the equilibrium toward dimer was much greater than the amount that is needed (∼10 μM) to saturate that site and it is thus unlikely that the high-affinity site is responsible for the Mg^{2+} effect. There is also a low-affinity Mg^{2+} binding site near the interface that forms a tetramer from two dimers (Jacobson et al., 1994), but that site is deleted in M15 β-galactosidase and would thus not play a role in the monomer−dimer equilibrium. It is also unlikely that the effect is due to ionic strength. The buffer used in these studies was quite concentrated (0.2 M Tris/0.2 M Tricine) and an ionic strength effect would have been achieved without adding only 20 mM Mg^{2+}.

The gel-filtration, native PAGE, and sucrose density experiments showed that there is a significantly higher dimer concentration in the presence of NaCl. The K_{diss} calculated in the presence of 150 mM NaCl was 0.4×10^{-7} M (compared to 2.5×10^{-7} M without NaCl). The shift toward dimer formation at the high concentrations of NaCl could be due to charge shielding and/or hydrophobic interactions. As stated previously, the buffer used in these studies was quite concentrated (0.2 M Tris/0.2 M Tricine) and an ionic strength effect would probably have been achieved already without the addition of more NaCl. β-Galactosidase requires quite high amounts of Na^+ or K^+ (about 100 mM) for full activity and it is possible that Na^+ binds to a specific but low-affinity Na^+/K^+ site and results in stabilization of the dimer. The location of this site was not established by the

three-dimensional structural study of this enzyme (Jacobson et al., 1994).

Thiol reagents had dramatic effects. The monomer−dimer equilibrium was shifted entirely toward dimer. It is also significant that the native PAGE dimer band was sharp and distinct. This indicates that little or no dissociation was occurring during the native PAGE runs. DTT had the same effect as did 2-mercaptoethanol but the concentration of DTT needed to cause dimer formation was lower due to the larger equilibrium constant for DTT reduction and the lower amount of disulfide present in DTT. Control experiments showed that reagents having structures similar to mercaptoethanol and DTT that do not contain sulfhydryl groups (e.g., ethanol and glycerol) did not have any effect on the monomer−dimer equilibrium. The dramatic effects of thiol reagents indicate that reduction of -SH groups is very important. Gregoret et al. (1991) have shown that the majority of the free sulfhydryl groups in proteins are actually involved in H-bonds and are important for structure. The thiols could prevent or reverse the formation of disulfides or other oxidized forms that could disrupt the structure. A search for Cys at the monomer−monomer interfaces was made using the structure determined by Jacobson et al. (1994). Jacobson et al. (1994) state that none of the Cys are nearer than 4.5 Å to the interface. Cys-825 is the sulfhydryl residue that is closest to the interface. Cys-914, -939, and -1021 are also reasonably near. Other Cys residues that are not as near to the interface could also be important for structure through long-range effects.

Increases in the amount of dissociation to monomer as the pH is raised can be explained by (a) deprotonation of cationic groups eliminating ion-pair attraction or (b) long-range electrostatic repulsions as the protein becomes more negatively charged. The unexpected increase in dimer present at pH 9.0 could be from the involvement of a residue with a pK_a of approximately 8.0 that could bring about a conformational change conducive to dimer formation. There are only three side chains (those of Lys, Tyr, and Cys) that could have pK_a values near 8.0, and of these, Cys is the only one with a pK_a that is normally about 8.0. The spectral difference titration experiments showed that the Tyr groups of β-galactosidase have pK_a values higher than 9.0 and are thus unlikely to cause the effect. In previous work using reductive alkylation with ^{13}C-enriched formaldehyde and NMR, Roth (1995) showed that each Lys group in β-galactosidase has a pK_a of 9.5 or higher. Therefore, Cys is the most likely residue involved. Sulfhydryl groups are known to readily oxidize if the pH values are above the pK_a values. The pH studies reported here were done in solutions without thiol reagents and it is possible that one or more of the sulfhydryl groups oxidizes readily when the pH is above the pK_a value and thereby promotes the formation of dimer.

The monomer−dimer equilibrium is reversible, and therefore, the reaction to form tetramer should be driven to completion by addition of α-peptide, even starting with monomer. The specific activities (after α-complementation in the presence of 20 mM Mg^{2+}, 145 mM NaCl, and 100 mM mercaptoethanol) of fractions collected over the complete elution profile were all similar and thus the formation of tetramer is indeed driven to completion even starting with monomer.

It is of interest that the optimal rates of the complementation reaction established by Langley and Zabin (1976) are similar to those that were found in this study for optimal formation of dimer. This implies, but does not prove, that the amount of M15 β-galactosidase that is present as dimer has an influence on the rate of the complementation reaction.

The complemented tetramer, once formed, does not dissociate readily to dimer or monomer under the various conditions studied. Only in the absence of NaCl was any dissociation to dimer noticed by gel filtration. There was no trace of any dissociation in a native PAGE experiment of complemented M15 β-galactosidase in which none of the three components that stabilized the dimer were present.

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