Thermal Denaturation of β-Galactosidase and of Two Site-Specific Mutants†

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ABSTRACT: The thermal denaturation of wild-type β-galactosidase and two β-galactosidases with substitutions at the active site was studied by kinetics, differential scanning calorimetry, electrophoresis, molecular exclusion chromatography, and circular dichroism. From the results, a model is developed for thermal denaturation of β-galactosidase which includes the reversible dissociation of ligands, reversible formation of an inactive tetramer, irreversible dissociation of the inactive tetramer to inactive monomers, and subsequent aggregation of inactive monomers to dimers and larger aggregates. Under some conditions, partial reversibility of the activity loss could be demonstrated, and several intermediates in the thermal denaturation process were trapped by quenching and observed by electrophoresis and molecular exclusion chromatography. The ligands Mg^{2+} and phenylethyl thio-β-D-galactoside increase the stability of β-galactosidase to heat denaturation by shifting the ligand binding equilibrium according to Le Chatelier's principle, thus decreasing the concentration of the ligand-free tetramer which can proceed to subsequent steps. Circular dichroism results indicated that β-galactosidase is dominated by β-sheet with lower amounts of α-helix. Large changes in secondary structure begin to occur only after activity has been lost. Single amino acid changes at the active site can have significant effects on thermal stability of β-galactosidases. Some of the effects result from increased thermal stability of the ligand-free enzyme itself. Other effects result from changes in ligand binding, but the magnitude of the resulting changes in stability is not related to the strength of ligand binding in a simple fashion.

Thermal denaturation of β-galactosidase (EC 3.2.1.12) has been used as a sensitive means of distinguishing between mutations of *Escherichia coli* which lead to changes in the amount of β-galactosidase produced and mutations which lead to changes in the amino acid sequence of the β-galactosidase molecules (Langridge, 1969). The primary structure of the 1023 amino acids in the 4 identical subunits of the enzyme from *Escherichia coli* is known (Fowler & Zabin, 1978; Kalnins et al., 1983), but structural information at the secondary and tertiary level is limited. Percentages of secondary structural types have been calculated from FTIR results (Arrondo et al., 1989), and several investigators have provided evidence which suggests that the enzyme consists of three domains (Lin & Zabin, 1972; Celada et al., 1978). Site-specific mutagenesis has recently been used to establish roles for Tyr-503 and Glu-461 in the catalytic mechanism of β-galactosidase, and mutations at positions 503 and 461 were shown to affect the thermal stability of the enzyme (Ring et al., 1988; Bader et al., 1988; Cupples et al., 1990).

In this paper, a model is developed for thermal denaturation of β-galactosidase based on the results of kinetic, differential scanning calorimetry, circular dichroism (CD) spectroscopy, electrophoresis, and molecular exclusion chromatography studies of wild-type β-galactosidase and β-galactosidases from two site-specific mutants. This model includes the reversible formation of inactive tetramers and irreversible dissociation of the inactive tetramers to monomers. The affinity of β-galactosidase for a divalent metal cation, Mg^{2+}, which activates the enzyme and for a tight binding competitive inhibitor, phenylethyl thio-β-D-galactoside (Van der Groen, 1973), has significant effects on the thermal stability of β-galactosidase, and their role is included in the model. Because β-galactosidase is a tetrameric enzyme which is easily isolated in reasonable quantities and which can be assayed at denaturing temperatures, this system can serve as a good model for the effects of both site-specific mutations and ligands (such as metals and inhibitors) on the thermal stability of oligomeric proteins for which unfolding is an irreversible process.

Materials and Methods

Materials. *o*-Nitrophenyl β-D-galactopyranoside (ONPG),[1] PNPG, PETG, and EDTA were from Sigma, and other chemicals were from Fisher or similar sources. Concentrations of these compounds were determined by weight. β-Galactosidase was purified [by a procedure similar to that of Brake et al. (1978)] from *E. coli* strain ML-308 for the wild-type enzyme, from strain JM108 transformed with plasmid containing the Y503F mutation, and from strain S185 transformed with plasmid containing the E461Q mutation. Preparation of the plasmid has been described (Bader et al., 1988; Ring et al., 1988). After purification of the β-galactosidases, the major bands on SDS–polyacrylamide gel electrophoresis for each enzyme contained more than 95% of the protein. Protein concentrations were determined by the absorbance at 280 nm using an extinction coefficient of 2.09 cm^2 mg^{-1} (Huber et al., 1979).

Enzyme Assays. β-Galactosidase activity was determined by following the increase in absorbance at 420 nm using an extinction coefficient of 2.65 cm^{-1} mM^{-1} for ONPG in an assay buffer consisting of 30 mM TES containing 145 mM NaCl and 1.00 mM $MgSO_4$ neutralized to pH 7.0 with HCl. Some kinetic measurements were done with PNPG as the substrate

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[1] Abbreviations: ONPG, *o*-nitrophenyl β-D-galactopyranoside; PNPG, *p*-nitrophenyl β-D-galactopyranoside; PETG, phenylethyl thio-β-D-galactopyranoside; EDTA, ethylenediaminetetraacetic acid; DSC, differential scanning calorimetry; T_x, the temperature required for x percent activity loss (usually T_{50}, which was used to indicate the temperature required for 50% activity loss); T_{loss}, the set of T_x values over which activity is lost; T_{max}, the temperature of the maximum of the DSC curve.

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in the same TES assay buffer using an extinction coefficient of 7.20 cm^{-1} mM^{-1} at 420 nm. For T_{loss} determination, the temperature of the cell block was increased at a rate of 20 K/h. At various time intervals, samples were removed and quenched by diluting the enzyme samples into ambient TES assay buffer. In a few cases (as indicated under Results), the same phosphate buffer in which the samples were heated was used as the quenching buffer.

In some cases, activity assays were conducted at the denaturing temperatures. Such assays were done with ONPG in 20 mM sodium phosphate buffer at pH 7.50 containing 0.50 mM 2-mercaptoethanol with either 1.00 mM $MgSO_4$ or 1.00 mM EDTA. In a few cases, studies were done with 2.00 mM $MgSO_4$ and 1.00 mM EDTA present. Changes of pH with increasing temperature for this phosphate buffer were negligible. The absorbance at 420 nm was continuously recorded, but samples were also removed at time intervals and quenched into ice-cold phosphate buffer. (Ice-cold rather than ambient buffer was used in this case because it was necessary to store the samples without excessive buildup of the reaction product in the 1–3 h of storage before the 25 °C assays could be done.) Percentages of activity at each denaturation temperature were determined from the slopes of the absorbance curve at 1-min intervals, which were compared to the changes in absorbance expected at each temperature if no activity had been lost. Correction was made for temperature effects on the absorbence of ONP by determining relative extinction coefficients over the temperature range 10–70 °C at 10 °C intervals and empirically interpolated by fitting to a quadratic equation (the regression coefficient was 1.00 for seven points). The activities expected for a 100% active enzyme at denaturing temperatures were found by empirical extrapolation of Arrhenius plots. Since k_{cat} for β-galactosidase is a combination of rate constants, it need not strictly fit a linear Arrhenius plot; thus, the slight deviations from linearity observed were accounted for by empirically fitting to quadratic equations.

Differential Scanning Calorimetry. Calorimetric experiments were performed on a Hart Scientific Model 701 differential scanning calorimeter. Typically, 1.00 mL of a 1.00 mg/mL sample was used in the 20 mM sodium phosphate buffer at pH 7.50 containing 0.5 mM 2-mercaptoethanol with either 1.00 mM $MgSO_4$ or 1.00 EDTA present. Higher ionic strength buffers and higher concentrations of 2-mercaptoethanol gave a large exothermic transition within the temperature range of denaturation which corresponded to the formation of a visible precipitate. Unless otherwise stated, a scan rate of 20 K/h was used. Base lines for the heat capacity curves were established from a repeat scan after the protein had been denatured (since no reversibility was found) and from a second scan on a carefully matched buffer sample. The temperature of the heat capacity maximum was designated T_{max} in each case. The areas under the heat capacity curve below and above T_{max} were approximately equal, and, therefore, T_{max} is quite close to the true melting temperature. The T_{max} and ΔH_m values reported are averages from at least two separate scans for wild type and Y503F-β-galactosidase, but only one scan under each set of conditions was done for E461Q-β-galactosidase because of limited amounts of this enzyme. The largest standard deviations obtained were 0.2 °C for T_{max} and 1.3 J/g for ΔH_m.

Binding Studies. Competitive inhibition constants with PETG as the inhibitor (using ONPG or PNPG as substrates) were determined according to the method of Huber and Gaunt (1983) which takes into account the fact that some inhibitors are also acceptors in the transferolysis reaction of β-galactosidase. For normal β-galactosidase, both substrates gave the same value of K_i, and so for β-galactosidase obtained from the mutants, only PNPG was used. Values of the standard free energy of binding ($\Delta G°$) were calculated from the K_i values.

To determine the dissociation constants for Mg^{2+}, the kinetic method of Dixon and Webb (1964) was used in a slightly modified form which took into account the fact that there is a low level of activity present in the Mg^{2+}-free enzyme. This kinetic method relies on the greater activity of the enzyme–metal complex as an indicator of the amount of enzyme with metal bound. Thus, at a Mg^{2+} concentration equal to K_D (where 50% of the enzyme molecules would have Mg^{2+} bound), the specific activity was assumed to be halfway between the values expected for the metal-free enzyme and the metal-saturated enzyme. Experiments were conducted at 15, 25, and 35 °C in sodium phosphate buffer with various concentrations of $MgSO_4$ and/or EDTA. The instability constants of Sillen and Martell (1964), interpolated to the correct temperature, were used to calculate the free Mg^{2+} concentration. The enzymes were preincubated for at least 2 h at each temperature for each concentration of Mg^{2+} before assaying so that equilibrium could be established. Hill plots at each temperature were used to test for cooperativity and to calculate the dissociation constants for Mg^{2+}.

Determination of Percentage Tetramer. The percentage tetramer was determined on samples cooled and diluted into the sample buffer by separating the tetramer peak from smaller (and larger) moleculer weight peaks on a Superose 12 molecular exclusion column or using native PAGE at pH 7.5 or 8.8. From the molecular exclusion column, the area under the tetramer peak was proportional to the peak height since the shape and width at half-height were constant as the tetramer peak decreased. Gels from the native PAGE were scanned after Coomassie blue staining, and the urea under the peaks was determined. In order to better visualize the weak bands on the gels, Coomassie blue staining was followed by AgCl staining. Quaternary structure was monitored without quenching by applying enzyme samples to a water-jacketed Superose 12 column heated to denaturing temperatures so that the various moleculer weight species could be formed and separated on the column without even having been cooled or diluted.

CD Spectra. CD spectra were measured on a JASCO J-500A spectropolarimeter calibrated with androsterone according to the manufacturer's specifications [0.05% (w/v) androsterone/dioxane has an ellipticity of 192.4 mdeg in a 1.0-cm cell at 304 nm]. Spectra were recorded in 1.00- or 5.00-mm water-jacketed cells at β-galactosidase concentrations of 1.00 or 0.10 mg/mL. Mean molar ellipticities were calculated based upon the amino acid composition of β-galactosidase determined from the sequence (Fowler & Zabin, 1978). The percentage helix and empirical indication of percentage β-sheet were calculated from the mean molar ellipticities at 13 wavelengths between 240 and 210 nm according to the method of Siegel et al. (1980). In order to follow the changes in structure during heating, the molar ellipticity at 222 nm was recorded in a separate experiment in which β-galactosidase was heated at 20 K/h.

Results

Enthalpy of Denaturation of Differential Scanning Calorimetry. Typical DSC curves for β-galactosidase in the presence and absence of Mg^{2+} (i.e., 1 mM EDTA) are shown in Figure 1a,b. The transitions are not completely symmetrical, but the enthalpy under the whole transition was taken as ΔH_m without attempts to resolve the curve into its under-

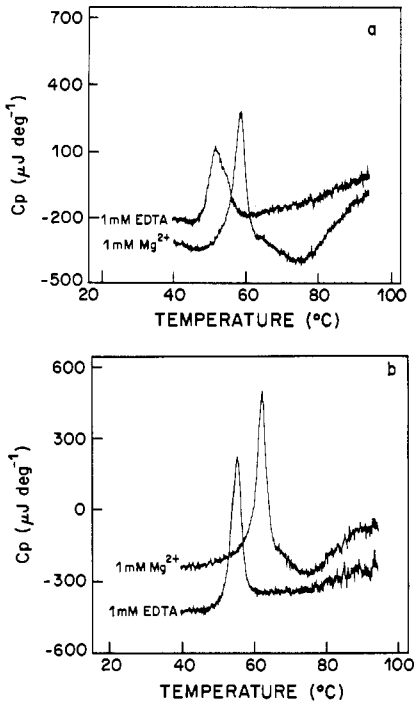


FIGURE 1: (a) Differential scanning calorimeter scans on 1.00 mL of 1.00 mg/mL β-galactosidase in 20 mM sodium phosphate buffer, pH 7.5, and 0.5 mM 2-mercaptoethanol in the presence of 1.00 mM Mg^{2+} or 1.00 mM EDTA. (b) DSC scans under the same conditions as above with 1.00 μM PETG.

lying components. No reversibility was found upon scanning from high to low temperatures or upon repetitive scanning after the first heating. In a few cases, samples were heated only to 80 °C and then cooled, to see if the irreversible changes were occurring only at high temperatures. No reversibility was, however, found.

In Figure 1a, the DSC transition is observed at higher temperature in the presence of Mg^{2+} than in the absence of Mg^{2+}. Similarly, in Figure 1b, which includes PETG, the DSC transition is observed at higher temperature in the presence of Mg^{2+} than in the absence of Mg^{2+}. The features on the high-temperature side of the main transition in the presence of 1.00 mM Mg^{2+} are variable and probably result from association of the denatured protein giving an exothermic transition with a minimum at about 77 °C. In the absence of both Mg^{2+} (i.e., 1.00 mM EDTA) and PETG, the DSC curves for wild-type β-galactosidase showed a broadening on the high-temperature side of the maximum (Figure 1a). Other scans in the absence of ligand both at higher and at lower protein concentrations failed to result in any significant further broadening. No broadening was observed in the Y503F- or E461Q-β-galactosidase DSC curves (not shown). The presence of PETG shifts the DSC curves to higher temperatures in both the presence and the absence of Mg^{2+} (i.e., compare Figure 1b to Figure 1a).

Table I tabulates the ΔH_m for all three of the β-galactosidases. In the absence of Mg^{2+} and PETG, Y503F-β-galactosidase has the same heat of melting as the wild type, but E461Q-β-galactosidase has a slightly higher heat of melting than either of the other two enzymes. The presence of 1.00 mM Mg^{2+} increases ΔH_m for wild-type β-galactosidase by 5.7 J/g and for Y503F-β-galactosidase by 2.6 J/g, but there was no increase for E461Q-β-galactosidase, and, thus, in the presence of Mg^{2+}, Y503F-β-galactosidase has a lower heat of melting that the wild type, and E461Q-β-galactosidase has a lower heat of melting than either of the other two enzymes.

Table I: Enthalpy of Melting (ΔH_m, J/g) for Wild-Type β-Galactosidase and Y503F- and E461Q-β-Galactosidases[a]

	wild type	Y503F	E461Q
1 mM EDTA	17.7	18.0	18.8
1 mM Mg^{2+}	23.4	20.6	18.8
1 mM EDTA + 1 mM PETG	20.7	19.2	ND
1 mM Mg^{2+} + 1 mM PETG	>21.8	>20.9	ND

[a] The values tabulated in the presence of both Mg^{2+} and PETG are only lower bounds as discussed in the text. The enthalpy of melting for E461Q-β-galactosidase was not determined (ND) with PETG present.

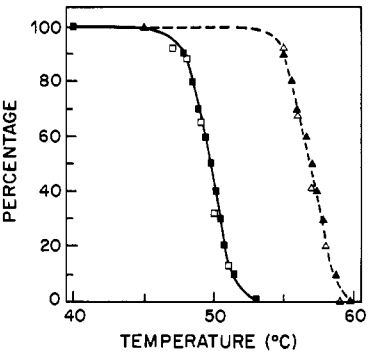


FIGURE 2: Percentages of activity and percentages of tetramer as a function of temperature for wild-type β-galactosidase. Samples containing 1.00 mg/mL enzyme were heated at 20 K/h, and aliquots were removed at various temperatures and added to ambient buffer. Percentages of activity were determined by adding aliquots of the quenched samples to 2.00 mM ONPG in TES buffer at 25 °C. Percentages of tetramer were determined from laser densitometer scans of native PAGE gels run at pH 7.5. The square symbols are in the absence of Mg^{2+} (1.00 mM EDTA), and the triangular symbols are in the presence of 1.00 mM Mg^{2+}. Percentages of activity are indicated by filled symbols, and percentages of tetramer are indicated by unfilled symbols.

The inhibitor, PETG, increased the ΔH_m for wild-type β-galactosidases and Y503F-galactosidase in the absence of Mg^{2+}. The ΔH_m values reported in the table in the presence of both PETG and Mg^{2+} are only lower bounds because the exothermic transition from the aggregation which occurs under those conditions overlapped the endothermic transition, making accurate determination impossible. The actual values are probably larger.

Data from a single DSC scan for which the heating rate was 40 K/h gave ΔH_m values of 16.3 and 21.7 J/g for wild-type β-galactosidase in the presence of 1 mM EDTA and 1 mM Mg^{2+}, respectively. These values are only slightly smaller than the ΔH_m values found when the heating rate was 20 K/h. In a separate experiment, the ΔH_m for wild-type β-galactosidase in the presence of 0.10 mM Mn^{2+} was found to be 24.1 J/g, which is slightly higher than the ΔH_m found in the presence of 1.00 mM Mg^{2+}.

Temperatures of Denaturation As Measured by Activity, Quaternary Structure, and DSC. Curves for the loss of activity of β-galactosidase and the amount of tetramer present in samples quenched by diluting at ambient temperature after the enzyme was heated to various temperatures are shown in Figure 2. Curves were obtained in the presence and absence of Mg^{2+}, and the T_{loss} values obtained were quite different in the two cases. Percentages of tetramer are not significantly different from the percentages of activity remaining either in the presence or in the absence of Mg^{2+}. This result was confirmed by gel exclusion chromatography of quenched samples (results not shown). Similar experiments for the mutated enzymes also gave percentages of tetramer which did not exceed the percentages of activity remaining. So, when quenched, the percentage of tetramer remaining equals the

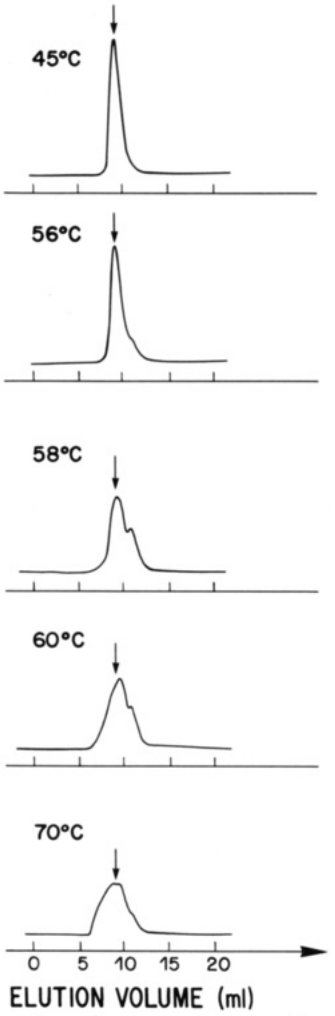


FIGURE 3: Molecular exclusion chromatography monitored by the absorbance at 280 nm of wild-type β-galactosidase heated to the temperatures noted on the frames of the figure in 20 mM sodium phosphate buffer, pH 7.5, with 1.00 mM Mg^{2+}. At the various temperatures, 100-μL aliquots were removed and added to 900 μL of buffer at room temperature. For chromatography, 300 μL of these quenched aliquots was applied to a Pharmacia Superose 12 column and eluted in the same buffer. The arrow in each frame is at 9.05 mL which is the volume where the β-galactosidase active tetramer elutes.

percentage of activity remaining in all cases.

Figure 3 shows the gel exclusion profiles of quenched samples of β-galactosidase which were taken as the temperature was increased. As the tetramer peak eluting from the molecular exclusion column began to decrease, a peak corresponding to a smaller molecular weight species began to form. At even higher temperatures, an intermediate molecular weight peak eluting between the tetramer peak and the smaller molecular weight peak appeared, first by shifting the maxima of the tetramer peak (58 and 60 °C) and then as a distinct peak (70 °C). Standards (results not shown) indicated that the smaller molecular weight peak was probably a monomer and that the intermediate molecular weight peak was probably a dimer. At 70 °C, very large molecular weight species migrating ahead of the original position of the tetramer are noticeable. Molecular exclusion chromatography without quenching conducted at high temperatures, so that both denaturation and separation occurred on the column, showed explicitly that the smaller molecular weight species formed first. The intermediate molecular weight species was also formed at denaturing temperatures without cooling but only appeared after the small molecular weight species was present

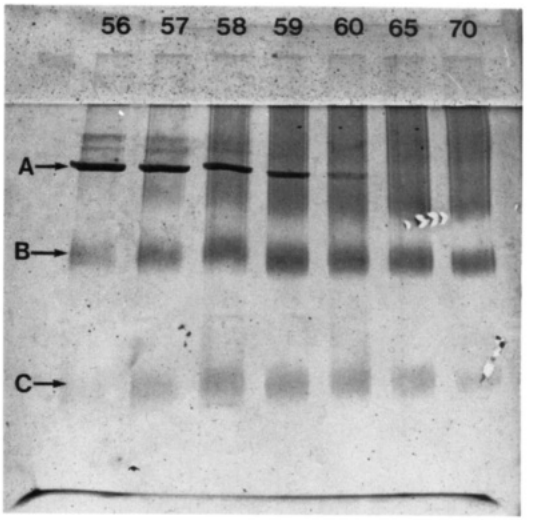


FIGURE 4: Native PAGE at pH 8.8 on 1-μL samples of the quenched aliquots prepared as described above. Lane 1 contained tracking dye only, and the temperatures at which the aliquot was taken are indicated for subsequent lanes. The gel was stained by the AgCl method which accounts for the prominence of the tetramer band.

in large amounts. However, the very large molecular weight species were not as noticeable on the chromatography conducted at high temperatures. The intermediate molecular weight species was more prominent in the presence of Mg^{2+} than in the absence of Mg^{2+}.

Native PAGE gels (of which a typical example is shown in Figure 4) also indicate that the smaller molecular weight species (band C) is probably a monomer and that the intermediate molecular weight species (band B) is probably a dimer. Although both monomer and dimer bands are observable at 56 °C, the monomer band in Figure 4 reaches a maximum and then rapidly declines above 59 °C whereas the dimer band only declines slowly above 65 °C. At even higher temperatures, the PAGE gels show broad aggregation bands as well as significant material at the boundary of the running gel. Native PAGE gels run at pH 7.5 gave the same tetramer, monomer, dimer, and aggregate bands as on the gel shown (which was run at pH 8.8), but the bands are not as well separated. Native PAGE of E461Q- and Y503F-β-galactosidase indicated that less of the dimer is present but monomer, dimer, and then higher aggregates are formed, in the same order as was observed for the wild type.

The average temperatures at which 50% of the original activity was lost are tabulated in Table II (as T_{50}) along with the T_{max} values obtained from DSC. In all cases, T_{50} was 1–3 °C lower than T_{max}. The temperatures required to give 90% loss of activity (T_{90}, not tabulated) were, however, essentially the same as T_{max}. In the absence of Mg^{2+}, E461Q-β-galactosidase had a higher T_{max} and T_{50} values than either wild-type or Y503F-β-galactosidase. Y503F-β-Galactosidase had about the same T_{max} and T_{50} as the wild-type enzyme. Thus, in the absence of Mg^{2+}, the relative order of stability; E461Q > Y503F = wild type, agrees with the enthalpy data. In the presence of Mg^{2+}, both of the β-galactosidases from the mutants had lower T_{max} and T_{50} values than the wild-type enzyme, which also agrees with their lower stability from the enthalpy data. However, Y503F-β-galactosidase had essentially the same T_{max} and T_{50} values as E461Q-β-galactosidase even though the enthalpy of melting for Y503F-β-galactosidase was slightly higher than that for E461Q-β-galactosidase.

Data from the single DSC scan for which the heating rate was 40 K/h gave T_{max} values of 54.5 and 60.5 °C for wild-type β-galactosidase in the presence of 1 mM EDTA and 1 mM

Table II: Temperature at the Heat Capacity Peak (T_{max}) and Temperature for 50% Loss of Activity (T_{50}) for All Three β-Galactosidases Determined at a Heating Rate of 20 K/h and an Enzyme Concentration of 1.00 mg/mL Except As Noted[a]

	wild type	Y503F	E461Q
1 mM EDTA			
T_{max}	51.6	52.5	55.7
T_{50}	49.8	49.5	53.2
T_{50} at 0.010 mg/mL	49.3	48.9	52.4
1 mM Mg^{2+}			
T_{max}	58.4	56.2	56.8
T_{50}	57.1	54.4	54.1
T_{50} at 0.010 mg/mL	57.6	54.5	54.1
1 mM EDTA + 1 mM PETG			
T_{max}	55.5	55.7	ND
T_{50}	53.1	54.3	55.6
1 mM Mg^{2+} + 1 mM PETG			
T_{max}	62.2	61.9	ND
T_{50}	60.5	60.6	58.2
stabilization by Mg^{2+}			
ΔT_{max} on adding Mg	6.8	3.7	1.1
ΔT_{50} on adding Mg	7.3	4.9	0.9
stabilization by PETG			
ΔT_{max} on adding PETG	3.8	5.7	ND
ΔT_{50} on adding PETG	3.4	6.2	4.1

[a] T_{max} values for the mutant β-galactosidase from E461Q were not determined (ND) in the presence of PETG. The changes in T_{max} and T_{50} upon addition of Mg^{2+} or PETG are tabulated as ΔT_{max} and ΔT_{50}.

Mg^{2+}, respectively. These temperatures are significantly larger than the T_{max} values found when the heating rate was 20 K/h. The T_{max} and T_{50} values for wild-type β-galactosidase obtained in the presence of 0.10 mM Mn^{2+} are 59.4 and 57.7 °C, respectively, which are slightly higher than the values found using 1.00 mM Mg^{2+}.

Influence of Protein Concentration and Ligands on the Temperature of Denaturation. In 1.00 mM Mg^{2+} and at protein concentrations below 1.00 mg/mL, an effect we presumed to be surface denaturation contributed significantly to total denaturation, but the presence of EDTA (even with excess Mg^{2+} present) eliminated the effect (unpublished results). In order, therefore, to determine T_{loss} in the presence of Mg^{2+} at low protein concentrations, both 1.00 mM EDTA and 2.00 mM Mg^{2+} were added to give a free Mg^{2+} concentration of approximately 1.00 mM. The T_{50} values determined for β-galactosidases from wild type and both mutants at low enzyme concentrations (values at 0.010 mg/mL are shown in rows three and six of Table II) demonstrated that T_{50} was independent of enzyme concentration.

The increases of T_{max} and T_{50} upon addition of Mg^{2+} or PETG, tabulated in Table II as ΔT_{max} and ΔT_{50}, are measures of the increased thermal stability provided by binding of the metal ion or the inhibitor molecule. Inspection of the table shows that the stabilization by Mg^{2+} follows the same order mentioned when discussing the enthalpy data: wild type > Y503F > E461Q. The stabilization by PETG follows the order Y503F > wild type = E461Q.

Binding of Mg^{2+} and PETG. Hill plots (not shown) indicated no cooperativity in the binding of Mg^{2+} for any of the β-galactosidases, in agreement with earlier reports for wild-type enzyme (Woulfe-Flanagan & Huber, 1978). The dissociation constants for Mg^{2+} (0.15 μM) and PETG (1.2 μM) binding to wild-type β-galactosidase at 25 °C are similar to those reported previously (Huber et al., 1979; Yde & De Bruyne, 1978). All three β-galactosidases bound Mg^{2+} more tightly as temperature was increased. Linear van't Hoff plots were obtained (not shown) and extrapolated to obtain the K_D values reported in Table III at 55 °C in order to compare the relative strengths of binding at a temperature at which denaturation

Table III: Dissociation Constants and Free Energy of Binding for Mg^{2+} and for PETG[a]

	wild type	Y503F	E461Q
Mg^{2+}			
K_D (nM)	7.6	6.6	160
$\Delta G°$ of binding (kJ/mol)	−51.0	−51.4	−42.7
PETG			
K_i (μM)	1.2	0.40	10
$\Delta G°$ of binding (kJ/mol)	−33.8	−36.5	−28.5

[a] The dissociation constant (K_D) for Mg^{2+} in sodium phosphate buffer, pH 7.50, was extrapolated to 55 °C as described in the text. The inhibition constant (K_i) for PETG was determined at 25 °C in pH 7.00 TES buffer with 1 mM Mg^{2+} present.

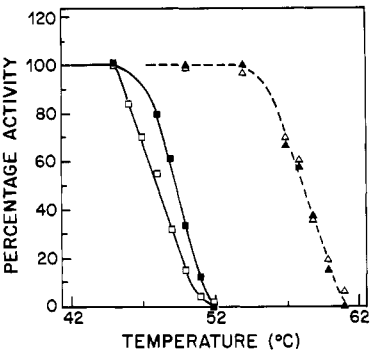


FIGURE 5: Percentage activity as a function of temperature for wild-type β-galactosidase assayed in the presence of 2.00 mM ONPG at denaturing temperatures which is indicated by unfilled symbols and after cooling to 25 °C which is indicated by the filled symbols. The square symbols are in 1.00 mM EDTA without added Mg^{2+}, and the triangular symbols are in the presence of 2.00 mM Mg^{2+} and 1.00 mM EDTA. The protein concentrations during the heating were approximately 0.0015 μg/mL in the presence of Mg^{2+} and approximately 0.13 μg/mL in the absence of Mg^{2+}. Aliquots were removed at various temperatures and added to ice-cold buffer. These tubes were warmed to 25 °C and the assays begun by adding 2 mM ONPG.

is actually taking place. The strength of the binding of Mg^{2+} follows the order Y503F = wild type ≫ E461Q, whereas the order of stabilization was wild type > Y503F > E461Q.

The $\Delta G°$ values for PETG binding to β-galactosidase at 25 °C indicate that the strength of the binding of PETG to the β-galactosidases follows the order Y503F > wild type > E461Q whereas the order of stabilization of the β-galactosidases was Y503F > wild type + E461Q.

Restoration of Activity in the Absence of Mg^{2+}. Figure 5 shows that, in the presence of 1.00 mM EDTA without added Mg^{2+}, β-galactosidase activities measured at denaturation temperatures were less than the activities measured at 25 °C after quenching. In the presence of Mg^{2+}, however, the activities at denaturation temperatures are the same as the activities measured at 25 °C after quenching. Other experiments (results not shown), in which the rate of activity loss was measured at constant denaturing temperatures, gave nearly first-order activity losses and confirmed that some activity could be regained upon quenching after the enzyme had been heated in the absence of Mg^{2+} but not in the presence of Mg^{2+}. The results with Y503F- and E461Q-β-galactosidase were different in that neither of these enzymes showed any restoration of activity either in the presence or in the absence of Mg^{2+}.

Experiments in which wild-type β-galactosidase had been heated at 20 K/h in the absence of Mg^{2+} until 28% of the activity remained at 49 °C showed that quenching into the TES assay buffer with 1.00 mM Mg^{2+} increased the activity to 49%. Quenching into phosphate buffer with 1.00 mM EDTA also increased the activity to 49%. Quick-cooling

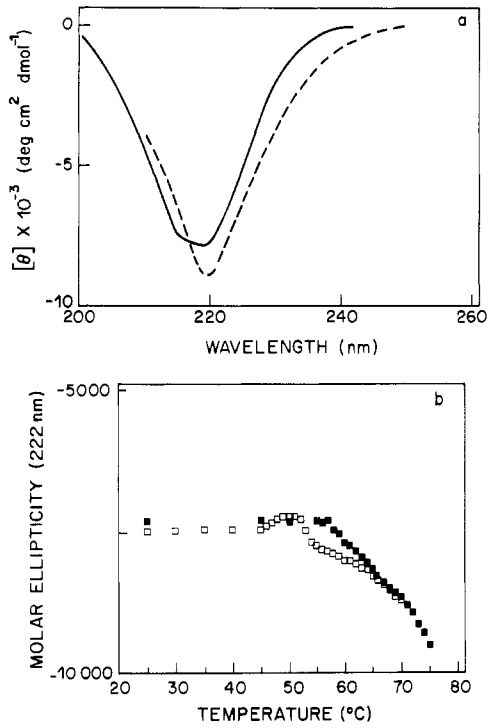


FIGURE 6: (a) Trace of the far-UV CD spectra for wild-type β-galactosidase in the presence of 1.0 mM Mg^{2+} in 20 mM sodium phosphate buffer, pH 7.5 at 25 °C (which is the solid line), and after heating to 75 °C (which is the dashed line). (b) Molar ellipticity at 222 nm as the temperature is increased in 1.0 mM Mg^{2+} which is indicated by the filled symbols) and in 1.0 mM EDTA (which is indicated by the unfilled symbols).

without dilution gave an increase of activity to 47%, but slow-cooling from 49 °C at about 1 K/min gave an increase of activity to only 37%. These experiments indicated that significant amounts of activity could be regained from wild-type β-galactosidase after heating in the absence of Mg^{2+} by rapid cooling and that it was not necessary to dilute or to have Mg^{2+} in the quenching buffer.

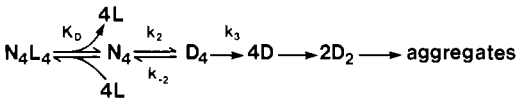
Denaturation Monitored by CD. The CD spectra of wild-type β-galactosidase in the low-wavelength region are recorded in Figure 6a. The percentage of α-helix in β-galactosidase calculated from the 25 °C spectrum is 14% with a standard deviation of 2%, and the percentage β-sheet calculated on the basis of the empirical equation of Siegel et al. (1980) is 48%. Upon heating above denaturing temperatures, changes in magnitude and small changes in shape were noted. The CD spectra in the aromatic region (250–300 nm) were very weak and could not be used to monitor tertiary structural changes on the time scale of heating.

In order to follow the changes of structure upon heating, the molar ellipticity at 222 nm was recorded while heating β-galactosidase (shown in Figure 6b). In the absence of Mg^{2+}, a very small decrease in the magnitude of the ellipticity is noticed in the region 46–51 °C. However, significant changes in the CD magnitude were not observed until the temperature had increased to 58 °C in the presence of Mg^{2+} and to 52 °C in the absence of Mg^{2+}. These temperatures are similar to the T_{90} values of 58.7 and 51.4 °C obtained in the presence and absence of Mg^{2+}, respectively.

Discussion

Proposed Scheme. A minimal scheme for the thermal denaturation of β-galactosidase which is consistent with all of the results is shown in Scheme I. L represents ligand (i.e., either Mg^{2+} or PETG). N_4L_4 and N_4 represent native β-galactosidase tetramers with and without ligand bound, respectively. D_4, D, and D_2 represent denatured (inactive) tetramers, monomers, and dimers, respectively. Since this is a minimal scheme, other intermediates, which were not indicated by our evidence, may intervene between those shown.

Scheme I



In no case (even with 1.00 mM EDTA) did the percentage of tetramer exceed the percentage of activity, and so in no case was the inactive tetramer trapped. The main evidence for the formation of D_4 is the restoration of activity in the presence of 1.00 mM EDTA with no Mg^{2+} added, and thus in other cases we postulate that very little of this intermediate exists (or perhaps none at all). There is no kinetic evidence for monomers forming directly from active tetramers and the inactive tetramers participating in a dead-end equilibrium with active tetramer, as was the case in the model proposed for urea denaturation (Wickson & Huber, 1970). D and D_2 were trapped upon quenching as shown by molecular exclusion chromatography and the native PAGE gels. The gel exclusion chromatography studies done at denaturing temperatures showed that all of the protein was found in the monomer peak before the dimer peak appeared, which established that D precedes D_2 and that the association to form the dimer occurs at denaturing temperatures and is not simply a result of cooling. However, cooling itself seems to enhance aggregation, and the larger aggregates may have formed only when the denatured samples are cooled.

Interpreting DSC Results for Irreversible Systems. Fukada et al. (1983) have developed a model and theoretical treatment for DSC results that applies to reversible unfolding of oligomeric proteins in which ligand molecules are released simultaneously with protein dissociation. The approximate applicability of reversible thermodynamic analysis of DSC data even when protein denaturation is irreversible overall has been defended on the basis of computer simulations of irreversible model systems and on the fact that the van't Hoff equation does operationally predict the effects of protein and ligand concentration on denaturation parameters (Edge et al., 1985, 1988; Manly et al. 1985). The simple reversible interchange of active and inactive tetramer yields an equation in which T_{max} is independent of total protein concentration (this is also applicable to T_{loss}), and consideration of the differential rate equations for the rates of the steps on Scheme I shows that since the dissociation of tetramer is the irreversible step, the denaturation temperature (either T_{max} or T_{loss}) is indeed independent of the total enzyme concentration. Fukada et al. (1983) have shown that T_{max} is determined by the ligand concentration and the temperature dependence of K_D (i.e., the enthalpy rather than the free energy of ligand binding). Inclusion of an irreversible step in the theoretical derivation is much more difficult, but it can be shown for Scheme I that the temperature of denaturation (either T_{max} or T_{loss}) depends on the ligand concentration and the relative sizes of K_D, k_2, k_{-2}, and k_3 as well as on their temperature dependence.

The one set of DSC results obtained at a higher heating rate established that the enthalpies of melting are not greatly influenced by the heating rate as would be expected since enthalpy is a state function and therefore independent of the path (i.e., reversible, or irreversible at various heating rates) if the initial and final states are the same. Earlier studies in this laboratory have shown that only active β-galactosidase binds

Mg^{2+} tightly (unpublished) and the divalent metal ion binding site on β-galactosidase is probably disrupted upon complete thermal denaturation. Although we did find there was more tendency to aggregate upon denaturation in the presence of Mg^{2+} (presumably because Mg^{2+} could enhance the association of the unfolded polyanionic protein), we were able to determine the enthalpy of melting at temperatures before aggregation became significant, except when both Mg^{2+} and PETG were present. Thus, the unfolded state would be essentially the same in the presence and absence of Mg^{2+}, and so the enthalpies of melting for the two cases can be compared. Significantly large T_{max} values were observed at the higher heating rate, which would also be expected because if the heating rate is large relative to the forward denaturation rate then temperatures above the transition temperature will be reached before sufficient denaturation occurs to give an observable transition (for either reversible or irreversible denaturation).

Effects of Protein Concentration and Ligands. Our data show that T_{loss} is, indeed, independent of the total β-galactosidase concentration and thus support Scheme I in which there is a reversible formation of an inactive tetramer followed by an irreversible dissociation of the tetramer. Renaturation of a tetramer with disrupted active sites to re-form the active tetramer would also be much more likely to occur than the alternative refolding and reassociation of dissociated subunits to form active tetramer, since the latter would require correct interactions between the subunits for reassociation as well as proper refolding within the chain.

The "stability" induced by the ligand can be explained as a result of Le Chatelier's principle as has been postulated for other systems by Fukada et al. (1983) and Edge et al. (1985). In the presence of added Mg^{2+} or PETG, the enzyme would initially be present primarily as N_4L_4 with only a very low concentration of N_4. Because the forward rate of step 2 is $k_2[N_4]$, the ligand-free active tetramer would proceed more slowly to subsequent steps in the presence of ligands, and, therefore, denaturation to D_4 would occur more slowly in the presence of ligands. Higher temperatures would be required to increase k_2 and thus provide a reasonable rate at which N_4 could proceed through step 2. Since k_3 would also increase with temperature, D_4 would rapidly proceed to irreversibly dissociate and thus would not be available for renaturation. However, in the absence of ligands, D_4 could build up and thus be available to refold and form active tetramer, accounting for the restoration of activity only observed upon quenching when Mg^{2+} is absent. Broadening of the DSC curve for the wild type in EDTA (with no Mg^{2+} or PETG added) also supports this proposal, because under these conditions the successive steps of the denaturation are partially separated so that the third step of Scheme I does not proceed quickly after the second step (i.e., k_2 is larger than k_3). For wild-type β-galactosidase, Mn^{2+} at 0.10 mM confers about the same stability as 1.00 mM Mg^{2+}, which is consistent with the approximately 10-fold tighter binding of Mn^{2+} (Huber et al., 1979).

Disruption of the Secondary and Tertiary Structure of β-Galactosidase. The secondary structural calculations based on CD spectra indicate that β-galactosidase is dominated by β-sheet (48%) with lesser amounts of α-helix. This agrees with the infrared spectroscopic results of Arrondo et al. (1979), although our CD results indicate that there is much less α-helix (14%) than did the IR results (35%). The small changes in molar ellipticity observed in the absence of Mg^{2+} between 46 and 51 °C may be monitoring small changes in secondary structure that occur concurrent with activity loss under these conditions. However, the CD data show that in both the presence and absence of Mg^{2+} the major changes in the secondary structure only began to occur after most of the activity had been lost. These major changes probably lead to the formation of an extended chain in a β-sheet-like conformation (Muga et al., 1988) which would account for the CD spectral changes observed.

The activity loss and the DSC results showed that T_{90} approximately equals T_{max}, and the ranges of the transition observed in DSC, which probably encompass the loss of tertiary and quaternary structure, seem to include only a small part of the secondary structural changes observed with CD. Thus, denaturation of β-galatosidase continues to occur after activity is lost and is not an all or nothing process for the individual enzyme molecules but must proceed through intermediate partially unfolded structures as is expected for protein denaturation (Shortle, 1989). Nearly all of the active sites have been disrupted when only about half of the enthalpy of melting has been absorbed as would be expected from the proposal of Tsou (1986) that the active sites of enzymes are often located in a region that is more flexible than the enzyme molecule as a whole and hence are more easily denatured.

Comparing and Contrasting the Wild Type and the Two Mutants. The relative stabilities of the mutants in the absence of ligands can be explained in terms of the proposed scheme if the ligand-free active tetramer of wild-type β-galactosidase and Y503F-β-galactosidase have about the same stability whereas the ligand-free active tetramer of E461Q-β-galactosidase is more stable than the other two so that higher temperatures are required to disrupt the active site and begin the process of denaturation for E461Q-β-galactosidase. In addition, the next step (from inactive tetramer to inactive monomer) must proceed more quickly for both mutants than for the wild type so that very little inactive tetramer would build up and thus, as was observed, no broadening of the DSC peak or restoration of activity would take place for the mutants (even in the absence of Mg^{2+}).

Because local interactions are modified by the active-site substitution of Gln for Glu-461, it is not surprising that its largest effect is on the denaturation step in which inactive tetramer forms active tetramer (i.e., active-site disruption). Removal of the negative charge when Glu-461 is replaced by Gln may decrease electrostatic repulsion between amino acid 461 and other nearby negatively charged side chains, thus leading to the observed greater stability of the active site for E461Q-β-galactosidase in the absence of Mg^{2+}. The higher temperatures required to form the inactive tetramer for E461Q-β-galactosidase account for much of the increase in the rate of the following step in which the inactive tetramer dissociates. Although local interactions are also modified by the active-site substitution of Phe for Tyr-503, the largest difference between the denaturation of Y503F-β-galactosidase and the wild-type enzyme is the increased rate of the tetramer dissociation step rather than the step which forms inactive tetramer by active-site disruption. Thus, this substitution seems to lead to interactions that do not affect the stability of the active site itself, implying that any hydrogen bonds formed by the Tyr hydroxyl do not greatly contribute to stability or that their loss in the mutant is compensated for by other, perhaps hydrophobic, interactions of Phe with surrounding amino acids.

The relative stability increases for the mutants upon binding the ligands, Mg^{2+} and PETG, do not exactly parallel the changes in affinity for these ligands (as measured by the

dissociation constant or calculated free energy of binding). This confirms the prediction of the theoretical treatment, discussed above, that a complete treatment of the stability conferred by a ligand requires consideration of more than just the strength of binding. Although the van't Hoff plots we obtained for Mg^{2+} binding appeared linear over the temperature range 15–35 °C, no attempt was made to determine the enthalpy of binding because the change in heat capacity upon binding may not be negligible.

Conclusion. Because of the difficulties involved in refolding and re-forming correct interactions between dissociated monomers even at temperatures which are not high enough to lead to covalent changes in the polypeptide chain, oligomeric proteins are even more likely to undergo irreversible denaturation than monomeric proteins. The methods and scheme developed in this study of β-galactosidase can be used as models of the effects of ligands and single site substitutions on irreversible denaturation of oligomeric proteins which may exhibit partial reversibility of the denaturation transition under some conditions. Enzymatic assays typically require much less protein than DSC experiments (as well as equipment which is more commonly available); therefore, information about protein concentration and ligand binding effects on stability are more easily obtained from T_{loss}. Since enzymatic assays are usually conducted at low protein concentrations whereas DSC data are collected at relatively high protein concentrations, in order to compare results from the two types of experiments, the effect of protein concentration must be explicitly considered.

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Registry No. PETG, 63407-54-5; Mg, 7439-95-4; β-galactosidase, 9031-11-2.

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