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Differential scanning calorimetry and fluorescence study of lactoperoxidase as a function of guanidinium-HCl, urea and pH

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Abstract

The stability of bovine lactoperoxidase to denaturation by guanidinium-HCl, urea or high temperature was examined by differential scanning calorimetry (DSC) and tryptophan fluorescence. The calorimetric scans were observed to be dependent upon the heating scan rate, indicating that lactoperoxidase stability at temperatures near T_m is controlled by kinetics. The values for the thermal transition, T_m , at slow heating scan rate were 66.8, 61.1 and 47.2°C in the presence of 0.5, 1 and 2 M guanidinium-HCl, respectively. Extrapolated value for T_m in the absence of guanidinium-HCl is 73.7°C, compared with 70.2°C obtained by experiment; a lower experimental value without denaturant is consistent with distortion of the thermal profile due to aggregation or other irreversible phenomenon. Values for the heat capacity, C_p , at T_m and E_a for the thermal transition decrease under conditions where T_m is lowered. At a given concentration, urea is less effective than guanidinium-HCl in reducing T_m , but urea reduces C_p relatively more. Both fluorescence and DSC indicate that thermally denatured protein is not random coil. A change in fluorescence around 35°C, which was previously reported for EPR and CD measurements (Boscolo et al. *Biochim. Biophys. Acta* 1174 (2007) 1164–1172), is not seen by calorimetry, suggesting that a local and not global change in protein conformation produces this fluorescence change.

Keywords

protein stability; denaturation

INTRODUCTION

Lactoperoxidase (LPO) is a 77.5 kD heme enzyme that is found in many body secretions including milk [1], saliva [2], earwax [3], and lung surfactant [4]. It catalyzes the oxidation of SCN^- by H_2O_2 to form $OSCN^-$; this product is a highly reactive oxidant that inhibits bacterial growth [5,6]. By catalyzing this reaction, LPO acts as the first line of defense against pathogens, preventing many of them from entering the mammalian body. As an extracellular protein found in many locations, LPO must be active under a variety of conditions, a fact that has clinical importance under some disease conditions. LPO is composed of a single polypeptide chain, and its heme is covalently attached to the polypeptide and 6 disulfide bonds contribute to the structure [7–9].

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In this study we are examining the effect of heating on protein unfolding using differential scanning microcalorimetry (DSC) and fluorescence with the goal of getting insight in the dynamic process of protein denaturation. Effect of extreme pH and addition of denaturants urea and guanidinium-HCl (Gdn-HCl) are compared. Analysis of calorimetric scans of LPO reveal that the observed melting temperature, T_m , depends upon scan rate, indicating kinetic influence on protein unfolding. Dynamics of unfolding was analyzed as described by Sanchez-Ruiz et al. [10]. The analysis is based upon a model originally presented by Lumry and Eyring in which native and unfolded states are in equilibrium, but a final denatured state is irreversibly produced from the unfolded state [11]. Assumptions made in the analysis procedure have been reviewed [12]. Denaturant lowers T_m , the value of C_p at T_m and the energy of activation for unfolding.

EXPERIMENTAL

Materials

LPO from bovine milk (EC 1.11.1.7) as a lyophilized powder, Gdn-HCl, N-acetyl-L-tryptophanamide, urea and Na phosphate were obtained from Sigma Chemical Company (St. Louis MO). The R_z value (ratio of absorption 412 nm to 289 nm) for LPO was 0.9. The resting LPO enzyme had absorption maxima at 412 and 280 nm. The ratio of the absorption at 412 to 280 nm was 0.783, consistent with the extinction of Trp and heme.

Water used for all solutions was first deionized using Millipore reverse osmosis and then glass distilled. Protein solutions were prepared by dissolving dry lyophilized protein into the buffer. Protein concentration was 1–3 μM ; the exact concentration was measured by the absorption at 412 nm, taking $112,000 \text{ M}^{-1}\text{cm}^{-1}$ as the molar extinction coefficient for the resting enzyme [13]. pH was measured with a glass electrode and an Accumet AB15 pH meter.

Spectroscopy

A Hitachi Perkin-Elmer U-3000 absorption instrument (Hitachi Instruments Inc., Danbury, CT) was used to take UV - visible absorption spectra.

Fluorescence emission spectra and intensity were measured with a Fluorolog-3–21 Jobin-Yvon Spex Instrument SA (Edison, NJ) equipped with a 450 W Xenon lamp for excitation and a cooled R2658P Hamamatsu photomultiplier tube for detection. Slit width was set to provide a band-pass of 2 nm. Measurements used conventional 90° geometry between excitation beam and detection of emission. Sample temperature was maintained by circulating water through the cell block. Cuvettes were made of quartz.

Calorimetry

Samples were degassed using MicroCal Thermovac degassing apparatus. The heat capacity was determined using MicroCal VP-DSC calorimeter; in this instrument, the temperature difference between reference and sample cells is compared and reference was always the solution in which the sample was dissolved. The thermograms were analyzed using MicroCal Origin software (analysis example shown in Figure 1). At fast heating scan rates, there is a small difference between sample temperature and the instrumental reading. This difference was corrected using the Origin software.

Analysis of calorimetric data

An assumption in the analysis is that the protein has two states, native (N), and unfolded (U), and that these forms interconvert at temperatures before irreversible denaturation occurs. When the heating rate is fast, the protein conformation does not always reach equilibrium, but by varying the rate of heating information on the dynamics of unfolding can be obtained. As

pointed out by Sanchez-Ruis et al. [10], for a two state irreversible denaturation process the rate constant of unfolding relates to the heating scan rate ν (°C K/min) by

$$k = \nu C_p / (Q_t - Q) \quad (1)$$

where C_p for the excess heat capacity, Q_t is the total heat change of the denaturation process and Q is the heat evolved at a given temperature. C_p^m is the apparent specific heat capacity at the observed maximum. The energy of activation, E_a , is obtained from different heating rates using the Arrhenius relationship: $k = A \exp(-E_a/RT)$.

The second equation of the kinetic model of Sanchez-Ruis and coworkers correlates the temperature T_m , at the maximum of the heat capacity change with heating rate ν :

$$\nu / T_m^2 = (AR/E) \exp(-E_a/RT_m) \quad (2)$$

In this case the $\ln(\nu/T_m^2)$ is plotted against $1/T_m$ yielding a straight line with slope $-E_a/R$.

The third equation presented by Sanchez-Ruiz et al. relates the heat produced with temperature as:

$$\ln(\ln Q_t / (Q_t - Q)) = (E_a/R)(1/T_m - 1/T) \quad (3)$$

For equation 3, a plot of the left hand term versus $1/T$ yields a line with slope $-E_a/R$.

In the final equation, the activation energy is obtained from the heat capacity at the maximum of the trace, C_p^m :

$$E_a = e R C_p^m T_m^2 / Q_t \quad (4)$$

where Q_t is the total heat change, as defined above and $e = Q_t / (Q_t - Q)$.

RESULTS

DSC of LPO

An example of data from DSC scans is shown on Figure 1A. An increase in heat capacity is seen with maximum at 66.8 °C in sample containing LPO relative to the reference, which contained 0.5 M Gdn-HCl at pH 5 without LPO. The method described by Sturtevant [14] was used to extrapolate the base-line as illustrated in Figure 1B.

The final data manipulation is shown in Figure 1C. The thermogram is corrected for base-line drift and the y axis is expressed as kJ/mole/°K.

Melting of LPO in the presence of Gdn-HCl and urea

The melting of LPO in the presence of the two commonly used denaturants, Gdn-HCl and urea, was examined. Thermal melting profiles are shown in Figure 2A–D for LPO at Gdn-HCl concentrations of 0, 0.5, 1 and 2 M for different heating scan rates. All parameters derived from these experiments are given in Table 1.

Focusing on Figure 2, it is apparent that the observed T_m is a function of Gdn-HCl and heating scan rate. For all denaturant concentrations the temperature of melting depends upon the heating scan rate. As denaturant concentration increases, T_m and C_p^m decrease.

In Figure 2D inset, value of T_m is given as a function of Gdn-HCl. Over this concentration range T_m linearly decreases with Gdn-HCl ($R^2 = 0.98$ to 0.99). However, for LPO the values extrapolated to zero denaturant are different from the experimental values; the extrapolated value is 73.7°C , whereas the experimental value is 70.2°C . Boscolo et al. [13] report that in the absence of denaturant, aggregation occurs. A negative heat capacity is expected for aggregation [15]; this can produce an apparent decrease in the transition temperature. In fact, at temperatures above the transition the baseline was below that before the transition without denaturant. Additionally, when the scans were repeated at higher protein concentrations, there was visible evidence of turbidity, proving aggregation.

The dependency of the derived experimental values upon heating rate shows that the protein macromolecule is not in thermal equilibrium during the temperature excursion. Using the model of Sanchez-Ruis et al. [10], the data collected at different heating rates were analyzed for E_a . In Figure 3A the data are plotted according to Equation 1; it can be seen that there is deviation from linearity at high temperature. The data from the lowest temperatures were used to fit to a line. The temperatures where k , the rate of unfolding, is equal to $1/\text{sec}$ are 69.5 , 64.4 and 53.1°C for 0.5 , 1 , and 2 M Gdn-HCl, respectively. These values are close to the value of T_m observed for the slowest heating scan rate (Table 1).

Data plotted according to Equation 2 are shown in Figure 3B. Figure 3C shows data plotted according to Equation 3. In general, the apparent E_a is lower for slower thermal scan rate. The value of C_p at the melting temperature in the absence of denaturant is lower than the extrapolated form (Table 1), and this is expected if aggregation was distorting the melting profile.

Figure 4A shows DSC scans in the presence of 1.0 and 2.0 M urea. Increasing urea decreased C_p^m and T_m . A scan at 4.0 M urea was not successful because the change in heat capacity was too low to be reliably obtained. Figure 4B, 4C and 4D present the data plotted according to Equations 1, 2 and 3.

As seen in Table 1, the trend is that under conditions where T_m decreases, ΔE_a decreases.

pH influence of LPO determined by DSC

In Figure 5, calorimetric traces observed at various pH's and at a heating scan rate of 0.5 and 1.5 deg/min are presented. The solution contained 1 M Gdn-HCl; similar pH dependence was observed for 0.5 M Gdn-HCl. The data taken at different thermal scan rates were analyzed by equations 1, 2 and 4, and the values from least squares analysis of the low temperature data are given in Table 2. At pH values between 5 to 8 , ΔH , T_m and E_a are weakly dependent upon pH.

pH influence of LPO determined by fluorescence

Fluorescence spectra are sensitive to solvent exposure; a red shift occurs when Trp goes from a hydrophobic to aqueous environment [16]. Spectra of indole group in hydrocarbon and in water and of the Trp of LPO below and above the melting temperature are shown in Figure 6B. Fluorescence spectra of LPO shift with protein denaturation by temperature [13] or pH and denaturant [17].

The fluorescence intensity ratio's, $I_{320\text{nm}}/I_{360\text{nm}}$, are plotted in Figure 7. The fluorescence spectra of the model compounds in octane or water are nearly temperature independent. In

contrast, LPO shows a shift at the melting temperature. A small transition, indicated by arrow, was noted at ~38°C. A transition at ~40°C was also observed for LPO using circular dichroism and spin label probe [13]. This small inflection was observed at all pH's, but was not detected in the DSC scan whereas the major unfolding event is seen by both DSC and spectroscopy. The DSC scans were repeated at 5 times the LPO to see whether a pre-transition could be detected, however none was observed.

It can be noted that the fluorescence of folded LPO is not as blue shifted as the Trp derivative in hydrophobic phase; the x-ray structure shows some Trp exposed to solvent. The fluorescence of the unfolded enzyme, above T_m , is not as red shifted as Trp in water; this would be consistent with some residual structure. LPO is stabilized by numerous cross-linkages by disulfide groups [9]; these cross-linkages would prevent a totally random coil structure for the denatured protein.

Comparison of activation energy, fluorescence and activity

In summary of the pH dependency, Figure 8A shows plots of T_m measured by fluorescence and DSC. They show the same temperature profile (compare closed circles with open squares for fluorescence and slow scan-rate DSC). The same pH dependency is also seen with varying heating scan rate (open and closed squares). Although absolute values for T_m change with thermal scan rate and Gdn-HCl concentration, all profiles exhibit the same dependency on pH. The data from Boscolo et al. [17] is also presented, showing agreement with previously reported work.

Figure 8B shows the pH dependency of E_a . When normalized, E_a follows the same pH profile as T_m . LPO enzymatic activity has a maximum at pH 6 [18]. Therefore, its maximal activity coincides with maximal stability. However, the protein is stable at pH values lower and higher than the peak of activity. The redox potential also shows pH-dependency, consistent with at least two redox-linked groups [19].

DISCUSSION

Our study verifies previous results [13,18,20], that LPO, like the related plant peroxidases [21–23], is thermally stable relative to other proteins. Our data expand upon the results of these papers in that dynamics is also considered. Lactoperoxidase is used in skin products and also naturally found on the skin. From the data presented in the figures and tables, extrapolation regarding protein dynamics can be made for the highest “physiological” temperature. At pH 6 and 40°C, the rate constant is $1.3 \times 10^{-7} \text{ sec}^{-1}$. At pH 7, this value is 0.1 sec^{-1} and at pH 8, k is equal to $1.7 \times 10^{-4} \text{ sec}^{-1}$. Therefore, at pH of typical hot tubs, which are usually alkaline, within about an hour or hour and a half, a significant fraction of skin LPO would still be active.

In examining the data of Table 1 and 2, a striking result is that the values for T_m and ΔE follow the same correlations with respect to the presence of Gdn-HCl and changes in pH. The significance of the reduction of T_m relates to how the protein structure is maintained and this subject remains controversial. The hydrophobic effect for protein unfolding posits that proteins are stabilized by the exclusion of water from the hydrophobic core [24]. This generally is considered the major, but not only effect [25]. Other workers [26] point out that H-bonding effects could have a significant role in the stability of proteins.

Perhaps some insight can be obtained by comparing the two denaturants. At a given concentration, urea is less effective than Gdn-HCl in reducing T_m (Table 1). However, when C_p values are plotted against T_m , urea is more effective (Table 1 and Figure 9). Infrared studies show that Gdn-HCl has a large effect on water structure [27] and it was suggested that the arrangement of water around Gdn^+ facilitates the solubility of hydrophobic groups. Gdn^+ has 6 H-bond donor groups. Its H-bonding to the amide group would destabilize the enzyme. In

addition, guanidinium is planar; on its flat surface, water H-bonding to other water molecules would be favored. This would enhance the solubility of hydrophobic groups; this idea is consistent with the experimental evidence that Gdn-HCl increases the solubility of hydrophobic groups in water [28,29]. It can be suggested, then, that Gdn-HCl potentially has multiple effects on the protein – both by direct interactions and indirectly by changing water H-bonding network. The action of Gdn-HCl, whether by direct binding or indirectly through changing the nature of the solute, causes a decrease in the energetic barrier required to unfold the protein and/or raises the energy of the folded state. Its value is 0.294 with no Gdn-HCl and 0.173 with 2 M Gdn-HCl. All these data are consistent with a reducing of the cooperativity of unfolding. Urea, in contrast, does not significantly change water structure, as deduced by infrared spectroscopy [30,31]. It has long been suggested that urea directly binds to the amide group [32]. However we note that urea, like Gdn-HCl, increases the solubility of hydrophobic groups [33], and there may be more than one effect of this denaturant, too. But, if the major effect of urea is to bind to the amide group, then comparing water to urea binding to the amide group, the entropy of binding of urea to the amide group would be lower. When entropy is lower, the overall enthalpy of the transition would be reduced.

The pH dependency was studied by DSC and fluorescence and compared with literature values for activity. There is general agreement between the three measurements in terms of activity and stability. Under the same conditions, the value for T_m occurs at pH 6 (Figure 7). However, subtle differences are seen between the three measurements. The transition of melting is broader seen by fluorescence than by calorimetry (Figure 6). For all pH's a small pre-transition was seen at around 35°C in fluorescence but not detected by DSC. In as much as the fluorescence signal arises from many Trp, a change around one Trp would be enough to cause this small change. It is therefore apparent that, although information on the dynamics of the protein is seen by fluorescence, one needs to remember that changes that Trp monitors are local, and need not reflect the conformation of the whole protein. LPO has 14 Trp, located in all areas of the protein, as indicated in Figure 6A. Therefore, fluorescence changes cannot be attributed to a particular part of the protein, but is likely reflect the conformation of the whole protein. However, residues near the heme are quenched by energy transfer which occurs by a Forster mechanism [34]; therefore, in the native state, the emission is biased toward Trp residues located away from the heme.

From the fluorescence data it is apparent that the peptide does not totally unfold in the heat denatured state. LPO is a rather large heme enzyme, 77.5 kD. As noted above it has six disulfide linkages and the heme of LPO is covalently attached to the polypeptide by ester linkages through 1-methyl and 5-methylporphyrin substituents, preventing heme dissociation from the polypeptide chain even under extreme conditions. In contrast, protoheme is readily dissociated from the polypeptide chain of other peroxidases under mild acidic conditions in aqueous buffers [35]. The dependence upon Gdn-HCl concentration in unfolding for LPO is larger than for cytochrome *c*, another heme protein in which the heme is covalently attached [36]. The heme is a large hydrophobic group that stabilizes heme proteins. The iron is ligated to the polypeptide chain in both axial and distal sides in cytochrome *c* and removal of iron reduces the stability of the protein to Gdn-HCl denaturation [34]. Peroxidases have only one protein ligating site and the heme pocket contains water on the distal side (see Figure 6); indeed, LPO absorption spectra is sensitive solvent [37].

In conclusion, the extra-cellular protein LPO is thermally stable at the temperatures found in usual physiological conditions; it has a high heat capacity, and a high energy of activation for the thermal transition. Gdn-HCl and urea lower the heat capacity and the transition becomes less cooperative, as E_a becomes lower.

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Abbreviations used

DSC	differential scanning calorimetry
LPO	lactoperoxidase
Gdn-HCl	guanidinium hydrochloride

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Figure 1. DSC thermogram traces

A. Thin line: both reference and sample compartments contained 20 mM phosphate, pH 5 and 0.5 M Gdn-HCl. Thick line: reference compartment contained same buffer and sample compartment contained 0.16 mg LPO/ml buffer for a protein concentration of 2 μ M. B. Dark line: difference between sample and reference; thin line: extrapolation of base line as described by Sturtevant [14]. C. Base-line corrected and normalized for concentration to express excess Cp/mole/degree.

Figure 2. Excess heat capacity of LPO measured at different heating scan rates and conditions
 Heating scan rates were as follows: 1. 1.5 $^\circ\text{C/min}$, 2. 1 $^\circ\text{C/min}$, 3. 0.5 $^\circ\text{C/min}$ and 4. 0.25 $^\circ\text{C/min}$. Sample contained ~ 0.2 mg LPO/ml 20 mM phosphate, pH 6 and Gdn-HCl concentrations as follows: A. none, B. 0.5 M, C. 1.0 M and D. 2.0 M. Inset: T_m as a function of Gdn-HCl. Line is a linear regression best fit. Y intercept is 73.7 $^\circ\text{C}$. R^2 is 0.998.

Figure 3. Kinetic analysis plots of heat capacity changes of LPO

Data from Figure. 2 were plotted according to: A. equation 1, B. equation 2 and C. equation 3. Symbols are for different heating scan rates as follows: closed circles, 1.5 °C/min; open circles, 1.0 °C/min; closed squares 0.5 °C/min; and open squares, 0.25 °C/min. Colors are for Gdn-HCl concentrations as follows: black, 0.0 M Gdn-HCl; red, 0.5 M Gdn-HCl; blue, 1.0 M Gdn-HCl and green, 2.0 M Gdn-HCl.

Figure 4. Excess heat capacity and kinetic analysis

Sample was 0.2 mg/ml LPO in 20 mM phosphate, pH 6. A. 1 M (1 and 2) and 2 M (3 and 4) urea. Heating scan rate was 1.5 °C/min (1 and 3) and 0.25 °C/min (2 and 4). Data from A plotted according to equation 1 (B), equation 2 (C) and equation 3 (D). Blue is for 1.0 M urea and green is for 2.0 M urea.

Figure 5. Thermograms of LPO at different pH's

Heating scan rate: 1.5 $^\circ\text{C/min}$. A. Sample was 0.2 mg LPO in 20 M Na-phosphate and 0.5 M Gdn-HCl at pH's indicated. B and C. analysis by eq 1 at the indicated pH.

Figure 6. LPO and fluorescence

A. Structure of LPO (coordinates from PDB 2NQX). Red: heme; blue, Trp. B. Normalized fluorescence spectra of $\sim 1 \mu\text{M}$ LPO in 20 mM phosphate, pH 6 and 1 M Gdn-HCl at 20 °C (dark line, 2) and 70 °C (line 3). Spectra 1 is for indole in n-octane and spectra 4 is for N-acetyl-L-tryptophanamide in 20 mM phosphate, pH 6.

Figure 7. Fluorescence intensity ratios as a function of temperature

A. N-acetyl-tryptophanamide in cyclohexane (open squares) or water (closed squares). LPO in LPO in 20 M Na-phosphate, pH 6, and 1.0 M Gdn-HCl (closed circles). B. Open circles give the derivative of LPO fluorescence ratios from A. Solid line is data from calorimetry at a heating scan rate of 0.25 °C/min. C. Derivative of LPO fluorescence ratios at (1) pH6 (2) pH5.0 and (3) pH4.5 and (4) pH 3.0

Figure 8. Comparison of data

A. T_m vs pH as determined by fluorescence and calorimetry. All samples had 20 mM Na-phosphate pH 6.0. Closed circle: T_m at 1.0 M Gdn-HCl measured by fluorescence ratio; Open square: for LPO at 1.0 M Gdn-HCl from DSC with 0.25 °C/min scan rate; Closed square: for LPO at 1.0 M Gdn-HCl from DSC with 1.5 °C/min scan rate; Grey circles: for LPO in 0.5 M Gdn-HCl from DSC with 1.5 °C/min heating scan rate; Open circles: data adapted from literature [17].

B. E_a for denaturation of LPO in 0.5 M Gdn-HCl (closed circle) and 1 M Gdn-HCl (open circle) in 20 mM Na-phosphate with heating scan rate of 1.5 °C/min; data calculated from Equation 1.

Figure 9. Comparison of Gdn-HCl and urea effect on C_p and T_m
Data from Table 1.

Table 1

Thermodynamic and kinetic parameters for denaturation of LPO

Scan rate, deg/min	T _m	C _p ^m , kJ/mol/deg	E _a (eq.1) kJ/mol	E _a (eq.2)	E _a (eq.3)	E _a (eq.4)
No Gdn-HCl						
0.25	70.2 [73.7]	360 [461]	664 [730]	571 [681]	697 [721]	599 [686]
0.5	71.1					
1.0	72.1					
1.5	72.6 [76.0]					
0.5 M Gdn-HCl						
0.25	66.7	360	619	571	634	610
0.5	67.4	355	580		601	532
1.0	68.4	340	558		618	465
1.5	69.3	291	527		580	488
1 M Gdn-HCl						
0.25	61.0	309	559	527	552	492
0.5	62.1	308	515		532	465
1.0	62.9	273	497		534	401
1.5	63.6	266	491		531	436
2 M Gdn-HCl						
0.25	47.2	107	338	306	378	340
1.5	51.7	106	322		352	223
1 M Urea						
0.25	66.6	246	557	483	628	561
1.5	69.5	246	460		544	422
2 M Urea						
0.25	63.4	179	454	411	481	432
1.5	67.1	162	413		455	368

* Equation (eq.) numbers are given in Materials and Methods. Sample conditions: 20 mM Na phosphate, pH 6.0. Number in brackets are extrapolated values from the data with Gdn-HCl to no Gdn-HCl.

Table 2

Thermodynamic and kinetic parameters for LPO as a function of pH

pH	T _m (°C)	C _p ^m , kJ/mol/deg	E _a (eq. 1)	E _a (eq. 2)	E _a (eq. 3)	E _a (eq 4)
4.1	44.5	74	323	393	398	247
5.1	60.1	216	498	481	554	345
6	63.6	266	491	527	531	403
7	63.7	219	479	559	559	467
8	58.4	185	384	454	371	389
8.9	50.9	141	379	349	418	353

* Equation (eq.) numbers are given in Materials and Methods. Sample conditions: 20 mM Na phosphate, 1 M Gdn-HCl at indicated pH values. Heating scan rate was 1.5 deg/min.