GAPDH architecture at low guanidine concentrations: first derivative analysis of the descending slope of the UV absorbance peak

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Abstract *– Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the glycolytic housekeeping enzyme, exists as an asymmetric tetramer. We previously observed that GAPDH can appear as a dimer and in higher order structures which we proposed may be a decamer. The monomeric subunit contains two rather distinct domains, which are referred to as the nucleotide-binding domain and the catalytic domain. These two domains occupy the N- and C-termini, respectively. We examined the denaturation of GAPDH in the presence of low concentrations of the chemical denaturant, guanidine-HCl (0.5-1.5M GdnHCl). Full denaturation of proteins typically require approximately 6M GdnHCl. At various concentrations of denaturant, UV absorbance spectra were obtained. The GdnHCl-dependent changes in the descending slope of the UV absorbance spectra were further examined by computing first derivatives of this region and monitoring changes in first derivative spectral peak and trough as a function of GdnHCl concentration. The observations of multiphasic changes are consistent with a model that suggests subunit separation is followed by domain-domain separation prior to domain unfolding.*

Keywords: glyceraldehyde 3-phosphate dehydrogenase, UV absorbance spectra, guanidine-HCl, denaturation.

1 Introduction

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is an indispensible protein that plays a pivotal role in glycolysis, a vital energy-generating pathway in all human cells. The glycolytic pathway consists of two stages. The first stage coverts 1mol glucose to 2mols D-glyceraldehyde 3-phosphate (Glyc3P) in five enzymatic steps. Net energy is not generated until stage two, where 1mol Glyc3P is converted to 1mol pyruvate. The first enzymatic step of the second stage of glycolysis is catalyzed by GAPDH. The substrates are Glyc3P, inorganic phosphate (Pi) and NAD+, and the products are 2,3BPG and NADH. The reaction is an oxidative phosphorylation and involves a covalent intermediate between the substrate Glyc3P and an active site cysteine residue. The next reaction is catalyzed by phosphoglycerate kinase (PGK) that converts ADP to ATP, in which a phosphoryl transfer occurs from 2,3BPG to ADP. Therefore in the first two reactions of the second stage of glycolysis, both NADH and ATP are made and become available for cellular processes. Increasingly, this general event has become understood as having specific purposes. For example, synaptic vesicles are equiped to load neurotransmitter, but in order to do this the vesicles are first acidified by the activity of a proton pump. This proton gradient drives the uptake of neurotransmitter into the vesicle. The proton pump is a vesicular ATPase which derives its ATP efficiently from a GAPDH-PGK complex [1]. The nature by which a cytosolic soluble protein like GAPDH becomes membrane bound is unknown. We recently proposed that GAPDH, which is typically described as an asymmetric tetramer, can appear as a dimer and in higher order structures which we propose may be a decamer [2]. Interestingly, the presence of inhaled anesthetics, such as isoflurane, shifts the equilbirum of the oligomer states towards the decamer [2], presumably through modulation of NAD+/NADH binding [3]. GAPDH is rather unstable, forming turbid solutions over time and is easily denatured upon heat exposure, showing a Tm (temperature at which 50% of the proteins are denatured) of 54.7°C [4]. This intrinsic tendency towards disorder may enable GAPDH to achieve diverse interactions with binding partners and thereby participate in alternate functions, such as interactions with vesicles, receptors and cytoskeletal components [5,6]. In order to develop a better understanding of the dynamic properties of GAPDH, we examined the changes in folded states of GAPDH at low concentrations of a chaotropic agent, guanidine (GdnHCl). By focusing on the descending slope of the UV absorbance spectra, we were able to use a computational approach to understanding the early steps of protein unfolding in GAPDH.

2 Materials and methods

Materials. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from rabbit muscle [EC 1.2.1.12] was obtained from Sigma-Aldrich (G2267) and dissolved in a 50mM sodium phosphate, *p*H = 7.4, buffer containing 0.3mM EDTA that was prepared with deionized (Milli Q; 18.2 MΩ) water and passed through a 0.2μm nylon filter (Millipore Millex-GN) prior to analysis. Concentration was determined by absorbance at 280nm using the molar aborbance coefficient of

$149 mM^{-1}cm^{-1}$ [7]. Guandine hydrochloride (GdnHCl) was also from Sigma-Aldrich (G4505) and freshly prepared prior to each experiment.

Treatment of GAPDH with GdnHCl. Incubations were done at room temperature as follows: samples (0.6mL) of GAPDH (10μM) were mixed carefully with 0, 0.5, 0.75, 1.0, 1.25 and 1.5M GdnHCl directly in quartz cuvettes. Final buffer concentrations were 30mM sodium phosphate, *p*H = 7.4, and 0.2mM EDTA. After thorough mixing, samples were assayed immediately.

UV Absorption Spectroscopy. Absorbance spectra (240-340nm; bandwidth 2.0nm; interval 0.5nm; lamp change at 325nm; scan speed 145nm/min) were obtained for the samples using a GE Healthcare Ultrospec 4000 spectrophotometer. The deuterium lamp had less than 100hrs of use. A reference sample that contained identical buffer without GAPDH was used before each scan. Spreadsheet data of the spectra were transferred to SigmaPlot 11.0 for further analysis

Computational Analysis. Difference spectra were first determined and compared. Absorbances from the experimental samples (GAPDH treated with GdnHCl) were subtracted from the control spectra to generate difference spectra. The downslope of the 280nm peak of the original spectra was closely examined. The descending component of the spectra from 283nm to 310nm was used to get first derivatives. Since the data was acquired at intervals of 0.5nm, regression lines were computed using 3 contiguous data points successively. With one data point shifts, the regression lines were overlapping and progressed from 283nm to 310nm, using SigmaPlot 11.0 to obtain slopes, which represented the tangent first derivatives. Since this component of the spectra is a downslope, all regressions were negative. To simplify analysis, absolute values were used and multipled by 100, thus keeping all values positive. The resulting first derivatives were plotted against wavelength. The plots presented spectra that exhibit maxima and minima that were reliable observations. In the range of 286-300nm, a single trough was seen followed by a single peak. In order to determine the exact points that represent the maximum and minimum, we used the equation for computing the center of spectral mass (CSM), by integrating over the range that pertained to the peak and trough, respectively.

$$\mathrm{CSM} = \sum (\lambda A i)\, di \,/\, \sum A i\, di \qquad (1)$$
$$(i = 0.5\mathrm{nm})$$

where λ is the wavelength (nm) and A is the absorbance at that wavelength. The CSM for the spectral nadirs (or troughs) and zeniths (or peaks) changed as a function of GdnHCl concentration. These values were then plotted against concentration of chaotropic agent to assess phasic response and transitons over this range of agent.

3 Results

The UV absorbance spectrum for control GAPDH showed a pattern typically seen for globular proteins, exhibiting a peak at approximately 280nm (Figure 1A) representing the aromatic residues. The absorbance below 240nm was ignored in this study. The absorbance in the 280nm range is in part due to the contribution of the microenvironment surrounding the aromatic residues, particularly tyrosines and tryptophans. Upon addition of GdnHCl, the change in protein architecture affects the micorenvironment around these residues, altering the spectra as seen in Figure 1A. The spectral changes were slight but very reproducible.

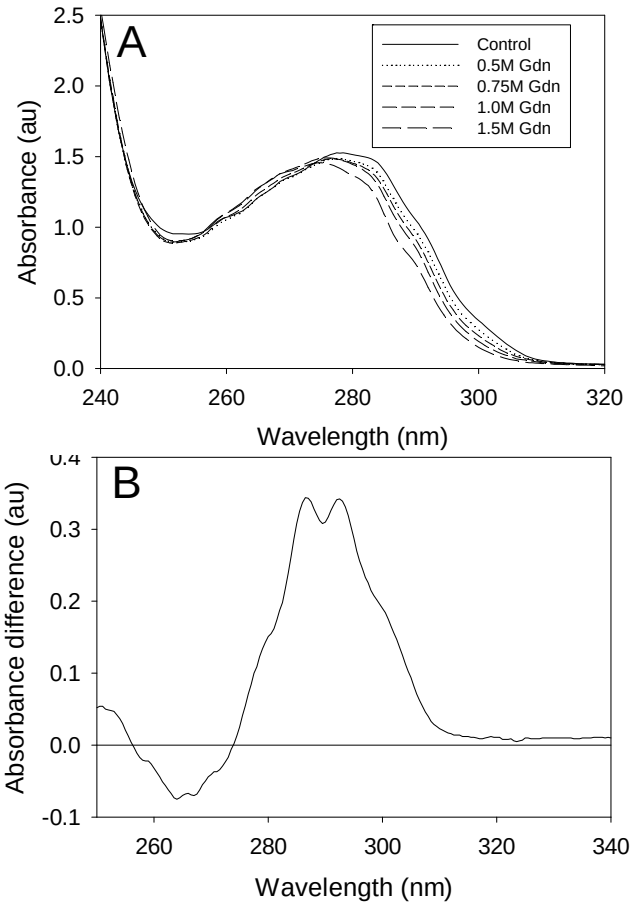

Figure 1: Spectral changes due to the chaotropic agent, GdnHCl. GAPDH (10μM) was incubated with various concentrations of GdnHCl. **A.** UV absorbance spectra in the 240 to 320nm range showing the effects of GdnHCl on the descending slope of the 280nm peak. **B.** The difference spectrum of control and GdnHCl(1.5M)-treated GAPDH.

Difference spectra were computed. Figure 1B presents the difference spectrum of control GAPDH and GAPDH-

treated with 1.5M GdnHCl. In this difference spectrum, we see the expected pattern of a trough with values below zero in the 260-270nm range and a peak (here, seen as a doublet) in the 285-295nm range.

The difference spectrum as shown in Figure 1B certainly provides information that is more readily visible than that found in the original spectra (Figure 1A). In fact, most studies that follow denaturation by absorption spectroscopy present difference spectra [8]. We extended this analysis to include an alternate approach, which involved examining first derivative conversions of the data.

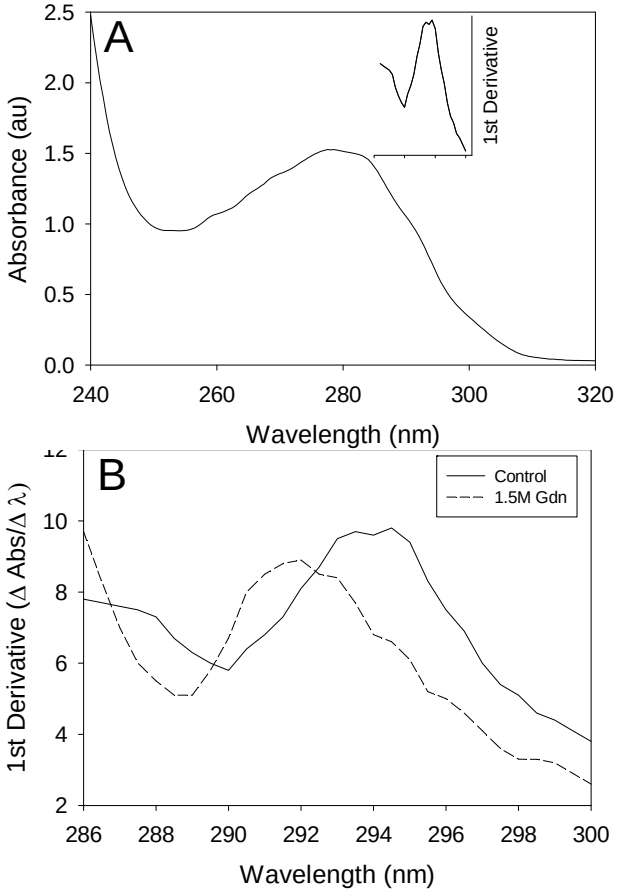

Figure 2: First derivative analysis of the descending slope of the 280nm peak. The data from Figure 1A were used to generate first derivatives. Control spectrum of untreated GAPDH was re-drawn in (**A**), showing the first derivatives over the 286-300nm region (**inset, A**), computed as described in *Materials and methods*. **B.** First derivatives were plotted as a function of wavelength for control and GdnHCl(1.5M)-treated GAPDH.

To pursue this aim of using an alternate approach to studying the effects of GdnHCl, we focused on the downward slope of the prominent 280nm peak from the original UV absorbance spectra. The first derivative spectrum in this range is juxtaposed to the original control spectrum in Figure 2A. The first derivative spectrum reveals a trough (or nadir) followed by a peak (or zenith). Control first derivative spectra were then compared with those of GdnHCl-treated samples. Figure 2B presents the data from control GAPDH against the spectra of GdnHCl(1.5M)-treated GAPDH.

Visibly one notices a shift in the spectra. Since a single spectrum includes a nadir and zenith, both can be quantified as a value (λ min and λ max, respectively). These values can be tracked as a function of GdnHCl treatment. The wavelength at which the peak or trough actually reaches its exact center point was determined mathematically using center of mass (CSM) calculation, with correction of the data around the trough to invert the tracing, making calculations feasible.

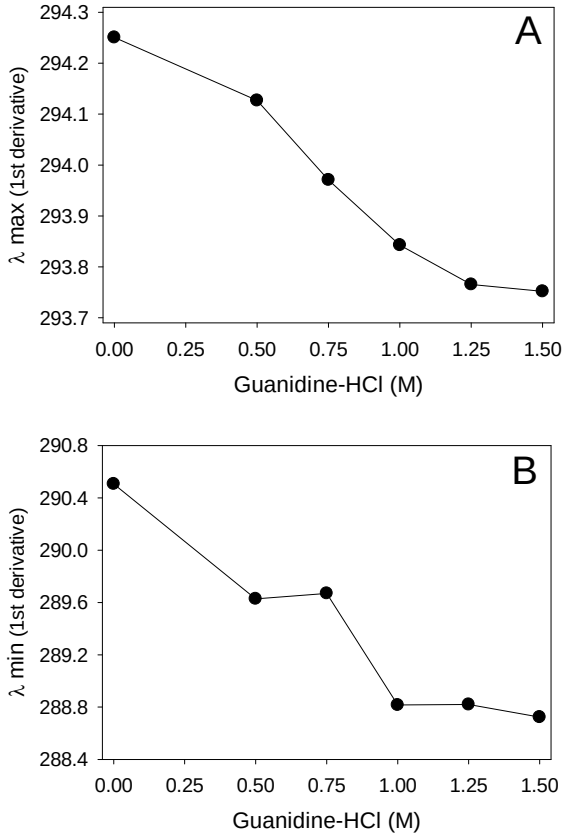

Figure 3: First derivative maxima and minima as a function of chaotropic agent, GdnHCl. First derivatives were initally computed for the downslope of the UV spectra; then the wavelengths representing zeniths (**A**) and nadirs (**B**) (λ max and λ min, respectively) were plotted as a function of GdnHCl concentration.

The wavelengths that represent the zeniths from the first derivative spectra were then examined as a function of concentration of chaotropic agent. The resulting plot (Figure 3A) exhibited two transition points: one at 0.5M and the other

at approximately 1.0M GdnHCl. We also observed that the nadir wavelengths plotted as a function of GdnHCl concentration showed three phases, an observation that was consistent with the computational data on wavelengths associated with the zenith.

Changes in secondary structure, which are indicative of domain unfolding, typically occurs at higher concentration of denaturant. For example, transferrin begins unfolding at approximately 1.6M GdnHCl [9]. The changes observed in this study, which looked at denaturant concentrations below 1.5M, were likely due to quartenary and tertiary changes that would occur prior to domain unfolding. It was previously shown that GdnHCl-induced dissociation and unfolding of a tetrameric enzyme exibits multiple phases over a 0.5 to 5M GdnHCl concentration range [10].

4 Discussion

In looking at the conformational effects of chemical chaotropic agents, a difference spectrum, such as Figure 1B, provides discrete information that is readily visible and potentially quantifiable as compared with the slight differences found in the original spectra (Figure 1A). In fact, most of the studies in the literature on chemical denaturation and absorption spectroscopy presents the results as difference spectra [8]. The intention of this study, on the contrary, was to use an alternate approach to improve data analysis of the original UV absorbance spectra. Converting the downslope of the spectra to first derivatives does increase the information found within the original data and reveals definable transition points that are seen as nadir and zenith values that reliably shift in response to low levels of chaotropic agent.

Previous studies show changes that occur prior to domain unfolding, which may be attributed to subunit and domain interfacial disruption. Using circular dichroism (CD) measurements of porcine serum transferrin at various concentrations of GdnHCl, two conformational transitions are evident [9], one at 1.6M and the other at 3.4M. Additionally, Fe^{2+}-binding is completely lost by 1.7M GdnHCl. There was a transition at approximately 1.2M, showing a slow trajectory of iron-binding loss from 0 to 1.2M GdnHCl followed by a faster trajectory from 1.2 to 1.7M GdnHCl. In this case, unfolding of secondary structure occurs above the CD-observable transition at 1.6M GdnHCl. Transferrin is a homodimer linked via a disulfide bond [11]. Each subunit consists of two domains, the N-lobe and the C-lobe with each lobe capable of binding a Fe^{2+} atom [12]. Each lobe is comprised of two subdomains. Upon more careful analysis of the authors' presentation of the iron binding data, we recognized that the process of iron-binding loss appeared to be multiphasic. The authors identified two phases that they attributed to sequential release of the two iron atoms. We think that the data shows three phases and that these phases represent changes in protein architecture that occurs at low GdnHCl concentrations. We propose that these architectural changes would correspond to iron binding loss. Their data (fig. 4b, in [9]) suggests that there are no changes to iron bound to transferrin from 0 to 0.2M GdnHCl, a rapid change from 0.2 to 0.5M, a then slower change from 0.5 to 1.2M, and then again a fast change from 1.2 to 1.7M, as visually inspected by us in the present study with acknowledgment that these values are estimates. First derivatives were calculated and were approximated to be 0.008 $\Delta Abs_{460}/\Delta GdnHCl(M)$, 0.004 and 0.008, respectively. These data may indicate that GdnHCl may be acting on disengaging the dimeric subunits first followed by separation of domains and then disruption of subdomain to subdomain interaction. While this is rather speculative and a re-analysis of existing data, the current study examined this proposed mechanism of the initial stages of denaturation using GAPDH. The term denaturation in this regard may be inappropriate, as these events likely do not alter the secondary structure given that the concentration of the denaturant is low. We think that the subunit-subunit interface, as well as the domain-domain interface, may represent an entry point for regulatory molecules that can alter the biological properties of GAPDH and providing its diversity of function. We recently proposed that GAPDH, an asymmetric tetramer, may dissociate to a dimer or reconfigure to higher order structures that may include a decamer [2]. Interestingly, inhaled anesthetics appear to shift the equilbirum away from the tetramer towards a dimer that may proceed to a decamer [2]. We think that these interfacial events may involve modulation of association with its cofactor [3]. The present study revealed that GAPDH's oligomeric structure may be labile.

The folding of proteins are considered to be based on the principle of a domain being the functional unit of folding [13], and that contiguous folding regions may be separated by linker regions [14]. Subdomains without extensive linker regions may likewise involve a heirarchical process of folding [15]. The mechanisms of unfolding likely proceed with these same principles. GAPDH is a multimeric protein, where each subunit is composed of two distinct domains. We proposed that using GdnHCl at low concentrations would differentiate the heirarchical levels of unfolding. Similar to these effects, we think that low levels of endogenous chaotropic compounds may modulate quartenary and perhaps even tertiary structure affecting biological function. Just as inhaled anesthetics appear to act at interfacial areas, GdnHCl's primary interaction may involve disruption of interfacial contacts.

The first derivative spectra reveal a trough (nadir) and peak (zenith) that may provide insight to changes in the microenvironments of the aromatic residues, differentiating the contribution by tyrosine and tryptophan residues. Our lab will continue to explore this possiblity. We intend to also explore the advantage of using second through fourth derivatives as was previously applied [16].

We observed that the wavelengths (that represent the

zeniths from the first derivative spectra of the downslope) decreased as a function of concentration of chaotropic agent. The resulting plot (Figure 3A) exhibited two transition points: one at 0.5M and the other at 1.0M GdnHCl, dividing the process into several phases. The first phase may involve separation of subunits from one another. This event would be initiated as GdnHCl was raised to 0.5M. The second phase from 0.5-1.0M GdnHCl may represent the separation of the domains from one another within the subunit structure. Above approximately 1.0M GdnHCl may involve secondary structure changes that are not as recognizable by these computational parameters. The shift of the zenith value appears to lessen and reach a limit value asymtotically. The nadir wavelengths plotted as a function of GdnHCl concentration also showed three phases, which was consistent with the zenith data.

5 Conclusion

The approach described in this study involved close inspection of the descending slope of the UV absorbance spectra, particularly examining the 283 to 310nm region, during exposure to low concentrations of GdnHCl. Molar concentrations of the chaotropic agent, such as 6M GdnHCl, completely unfolds most proteins. The effects of GdnHCl at concentrations below 1.0M remain poorly understood. Given the dynamic nature of GAPDH particularly that it exhibits multiple cellular functions with diverse binding partners, we proposed that subunit-subunit interfacial dynamics play a crucial role in GAPDH structure and function. Therefore, analysis of spectroscopic changes at low GdnHCl may reveal useful information regarding this dynamic feature. Conversion of UV spectra to first derivatives allows one to study the reliably appearing trough and peaks that exhibit quantal shifts. Our findings suggest that the GAPDH structure is easily perturbed and this intrinsic disorder, which likely resides at the interfacial regions, may contribute to functional diversity.

6 References

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