Analytical Biochemistry 389 (2009) 165–170

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Revisiting absorbance at 230 nm as a protein unfolding probe

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ARTICLE INFO

Article history:
Received 19 February 2009
Available online 24 March 2009

Keywords:
Protein stability
UV absorbance
230 nm
Conformational change
Unfolding kinetics

ABSTRACT

Thermodynamic stability and unfolding kinetics of proteins are typically determined by monitoring protein unfolding with spectroscopic probes, such as circular dichroism (CD) and fluorescence. UV absorbance at 230 nm (A_{230}) is also known to be sensitive to protein conformation. However, its feasibility for quantitative analysis of protein energetics has not been assessed. Here we evaluate A_{230} as a structural probe to determine thermodynamic stability and unfolding kinetics of proteins. By using *Escherichia coli* maltose binding protein (MBP) and *E. coli* ribonuclease H (RNase H) as our model proteins, we monitored their unfolding in urea and guanidinium chloride with A_{230}. Significant changes in A_{230} were observed with both proteins on unfolding in the chemical denaturants. The global stabilities were successfully determined by measuring the change in A_{230} in varying concentrations of denaturants. Also, unfolding kinetics was investigated by monitoring the change in A_{230} under denaturing conditions. The results were quite consistent with those determined by CD. Unlike CD, A_{230} allowed us to monitor protein unfolding in a 96-well microtiter plate with a UV plate reader. Our finding suggests that A_{230} is a valid and convenient structural probe to determine thermodynamic stability and unfolding kinetics of proteins with many potential applications.

The thermodynamic stability of a protein is a fundamental parameter that describes the energetic property of the protein structure. The experimental determination of thermodynamic stabilities requires the conformational transitions induced by elevated temperature or chemical denaturants to be monitored by conformational probes [1,2]. Spectroscopic probes sensitive to protein conformations are frequently employed for this purpose. Though less popular than circular dichroism (CD)[1] and fluorescence, UV absorption is also known to be sensitive to the conformational changes in proteins [3,4]. Absorbance at 280 nm by aromatic side chains is frequently used to monitor conformational changes in proteins as well as to determine protein concentrations.

Absorbance at 230 nm (A_{230}) is also known to be sensitive to protein conformation [4–9]. UV spectra of protein solutions usually show slopes, not peaks, at 230 nm. However, the difference spectra between folded and unfolded conformations commonly show a downward peak at 230 nm; i.e., unfolded proteins have lower A_{230} than folded proteins. Like absorbance at 280 nm, aromatic side chains, especially tryptophan, are known to contribute most to the change in A_{230} on protein unfolding [8,9]. The degree of the change in A_{230} on unfolding seems to depend on the number of aromatic amino acids that experience environmental changes, such as polarity changes from the protein interior to the bulk solvent [8,9]. Interestingly, the change in A_{230} on unfolding is typically ~10-fold greater than the change in A_{280} [8]. Though this probe has been discovered and investigated carefully in 1960s, the use of this wavelength has been quite rare. Though other spectroscopic probes have been frequently used to determine thermodynamic stability and folding kinetics of proteins, A_{230} seems to be mostly forgotten. However, as the development of high-throughput technology in recent years introduced UV transparent 96-well microtiter plates, A_{230} represents a sensitive and convenient probe to monitor protein unfolding in a high-throughput format.

Here we assess the applicability of A_{230} as a probe to investigate thermodynamic stability and unfolding kinetics of proteins. We used *Escherichia coli* maltose binding protein (MBP) and *E. coli* ribonuclease H (RNase H) as model proteins for this study. After confirming that the proteins show significant changes in A_{230} on unfolding, we determined the thermodynamic stabilities of the proteins by monitoring their unfolding in chemical denaturants by A_{230}. The validity of the method was checked by comparing the results with parameters determined by CD. We also monitored unfolding of MBP in 96-well microtiter plates by using A_{230}.

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[1] *Abbreviations used:* CD, circular dichroism; MPB, maltose binding protein.

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doi:10.1016/j.ab.2009.03.028

Unfolding kinetics of MBP and RNase H were also studied by A_{230} and the result was compared with the kinetic constants determined by CD. Based on these data, we evaluate the advantages and disadvantages of A_{230} as a probe for protein unfolding.

Materials and methods

Materials

MBP and RNase H were expressed and purified as previous reported [10,11]. Concentrations of proteins were determined spectrophotometrically in 20 mM sodium phosphate buffer (pH 6.5) containing 6.0 M guanidinium chloride (GdmCl) with extinction coefficients calculated with amino acid compositions [12]. Urea and GdmCl were from Shelton Scientific. A UV transparent disposable 96-well plate was obtained from BD Bioscience (San Jose, CA). $CaCl_2$ and KCl were from J.T. Baker (Phillipsburg, NJ).

Difference spectra between folded and unfolded proteins in the UV region

Proteins were diluted in 20 mM Tris–HCl buffer (pH 8.0) containing 50 mM NaCl to a final concentration of 50 μg/mL. For unfolded protein samples, GdmCl was added to 6.0 M. Before the measurement was made, samples were incubated at 25 °C overnight to reach equilibrium. The equilibrated solutions were then transferred to a UV transparent quartz cuvette and scanned from 220 to 250 nm at 25 °C by a Cary-3 Bio UV–visible spectrophotometer from Varian (Palo Alto, CA). The baselines of the buffer solutions were subtracted from the sample absorbance. The difference spectra were determined by subtracting the absorbance of folded proteins from the absorbance of unfolded proteins.

Equilibrium unfolding

MBP was diluted to the final concentration of 50 μg/mL in 20 mM Tris–HCl buffer (pH 8.0) containing 50 mM NaCl, 10 mM $CaCl_2$, and varying concentrations of urea [13]. RNase H was diluted to the final concentration of 50 μg/mL in 20 mM sodium acetate buffer (pH 5.5) containing 50 mM KCl and varying concentrations of urea [11]. Samples were equilibrated at 25 °C overnight. A_{230} of each sample was taken in a UV transparent cuvette or in a UV transparent 96-well microtiter plate, and 100 μL of each sample was used for the microtiter plate. Absorbance of the samples in the microtiter plate was measured in SpectraMax Plus384 UV–Vis plate reader from Molecular Devices (Sunnyvale, CA). To determine C_m and m values, A_{230} was fit to the equation

$$A_{230} = \frac{A_n - A_u}{1 + \exp(-\Delta G_{unf}^{\circ}/RT)} + A_u, \quad (1)$$

$$A_n = A_n^0 + s_n D,$$

$$A_u = A_u^0 + s_u D,$$

$$\Delta G_{unf}^{\circ} = m(C_m - D),$$

where A_n and A_u are the UV absorbance of folded and unfolded proteins, respectively, D is the denaturant concentration, m is the dependence of ΔG_{unf}° on the urea concentration, and C_m is the midpoint of the unfolding transition [1]. The global stabilities of proteins (ΔG_{unf}° in the absence of a denaturant) were determined by multiplying C_m and m values.

Unfolding kinetics

Unfolding of MBP was initiated by diluting a concentrated MBP solution to a final concentration of 50 μg/mL in 20 mM Tris–HCl buffer (pH 8.0) containing 50 mM NaCl, 10 mM $CaCl_2$, and varying concentrations of urea [14]. The change in A_{230} was then monitored at 25 °C by a Cary-3 Bio UV–visible spectrophotometer. Quartz cuvettes were sealed in order to prevent vaporization. Unfolding of RNase H was also initiated by diluting the protein to 50 μg/mL in 20 mM sodium acetate buffer (pH 5.5) containing 50 mM KCl and varying concentrations of urea. Unfolding kinetic constants were determined by fitting the changes in A_{230} to a first-order rate equation. The plots of log values of unfolding kinetic constants versus urea concentrations were fit to

$$\ln k_u = \ln k_u(H_2O) + \frac{m_u}{RT} D, \quad (2)$$

where $k_u(H_2O)$ is the unfolding rate constant of protein in the absence of urea, and m_u is the parameter showing the dependence of unfolding rate constants on the urea concentration.

Results

Change in A_{230} on unfolding of MBP and RNase H

The change in A_{230} on protein unfolding is known to be dependent on the change in the number of solvent-exposed aromatic amino acids, especially tryptophan. MBP and RNase H have eight and six tryptophan residues, respectively, which make them good candidates for application of A_{230}. To show that the proteins exhibit detectable changes in A_{230} on unfolding, we have determined the different spectra in the far UV region between folded and unfolded proteins. For comparison with circular dichroism, the protein concentrations were set to 50 μg/mL, which is within the range of protein concentration that is typically used for CD measurement. When unfolded in 6.0 M guanidinium chloride, both proteins show significant decreases in the far UV region (Fig. 1A). MBP shows a greater change in A_{230} than RNase H. The maximum change in absorbance was observed at 230 nm for MBP (ΔA = −0.123) and 231 nm for RNase H (ΔA = −0.075). When ΔA is converted to $\Delta\varepsilon$,

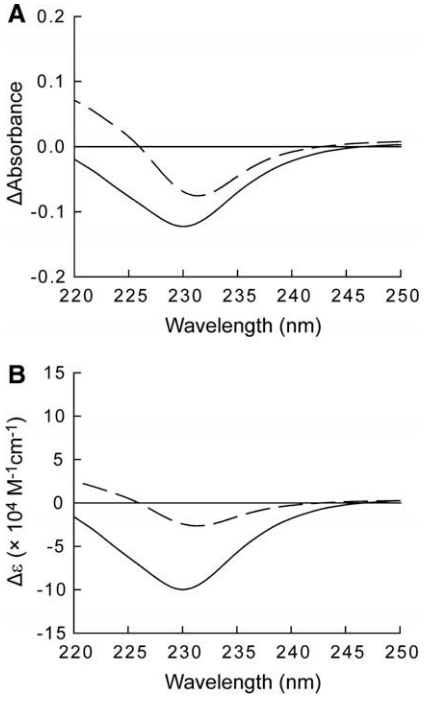

Fig. 1. The dependence of A_{230} on protein conformations. Difference spectra (A) and $\Delta\varepsilon$ (B) between folded and unfolded MBP (—) and RNase H (- - -). Unfolded proteins were prepared by incubating in 6.0 M GdmCl overnight.

the difference is even greater (Fig. 1B). The $\Delta\varepsilon$ values at 230 nm of MBP ($1.0 \times 10^5\ M^{-1}\ cm^{-1}$) are about fourfold greater than that of RNase H ($2.4 \times 10^4\ M^{-1}\ cm^{-1}$). It is noteworthy that $\Delta\varepsilon$ at 230 nm is not linearly proportional to the total number of tryptophan residues but to the change on unfolding in the number of solvent-exposed tryptophan residues. As previously reported [8], the changes in 230 nm region were much greater than the change in the 280 nm region (data not shown). With this concentration of protein (50 μg/mL), reliable detection of the changes in 280 nm on unfolding was experimentally challenging. The result clearly shows that A_{230} is a valid probe to monitor the unfolding of MBP and RNase H.

Determination of equilibrium unfolding by A_{230}

To test if A_{230} is reliable for quantitative determination of protein stability, we monitored A_{230} of MBP and RNase H in varying concentrations of denaturants. After MBP and RNase H were incubated in different concentrations of urea and GdmCl at 25 °C overnight, A_{230} of each sample was determined with a UV spectrophotometer. Fig. 2A shows the change in A_{230} of MBP and RNase H on unfolding in urea. Overall A_{230} measurements seem to be somewhat noisier than typical CD measurements under similar conditions. Under our experimental conditions the standard error from triplicate measurements of A_{230} with several different proteins was about 3% (data not shown). A_{230} of MBP shows a sharp transition near 3 M urea with monotonic increases in A_{230} before and after the transition. A_{230} of RNase H also showed a similar pattern. A transition was observed near 4 M urea with again monotonic increases in A_{230} before and after the transition. To evaluate the contribution of urea to these monotonous increases in A_{230} before and after the transitions, we have determined A_{230} of the buffer containing varying concentrations of urea without a protein. The absorbance of the buffer with urea showed a monotonous increase quite similar to the increase in A_{230} with RNase H and MBP before and after the transitions (data not shown). This similarity implies that urea (or any UV-absorbing impurity in urea) may be responsible for the monotonous baseline changes.

By fitting the change of A_{230} of MBP in Fig. 2A to Eq. (1), we determined the transition midpoint (C_m) of this protein to be 2.81 ± 0.01 M. We also determined the m value and unfolding free energy (ΔG_{unf}) to be 5.7 ± 0.3 kcal/(mol M) and 16.0 ± 0.8 kcal/mol, respectively. The C_m value of MBP was reported previously to be 2.83 ± 0.01 by CD under the identical conditions (Table 1), which is in excellent agreement with the A_{230} result. The m value by A_{230} is somewhat greater than the value determined by CD (5.2 ± 0.3 kcal/(mol M)) (Table 1).

We also determined the C_m value of RNase H to be 4.47 ± 0.03 M, the m value to be 1.7 ± 0.1 kcal/(mol M), and ΔG_{unf} to be 7.6 ± 0.5 kcal/mol. The C_m value is in good agreement with the value determined by CD (4.62 ± 0.01 M [11]) (Table 1). The m value (1.7 ± 0.1 kcal/(mol M)) is, however, less than the value determined by CD (2.1 ± 0.1 kcal/(mol M) [11]). This difference in m values is mostly responsible for the difference in the global stabilities determined by the two methods. A_{230} seems to be noisier than CD, which may result in greater error in the determination of m values. However, C_m values determined by A_{230} have good precision. The standard errors for C_m values from the regression were smaller than 1% for both proteins.

We also monitored the unfolding of MBP and RNase H in GdmCl with A_{230} (Fig. 2B). Unlike urea, the baselines (absorbance of the proteins before and after transitions) do not change significantly in varying concentrations of GdmCl. C_m and m values were determined by fitting the plots to Eq. (1) (Table 1). The C_m values again show good precision. The errors in the stability determination mostly result from the statistical uncertainty in the determination of m values.

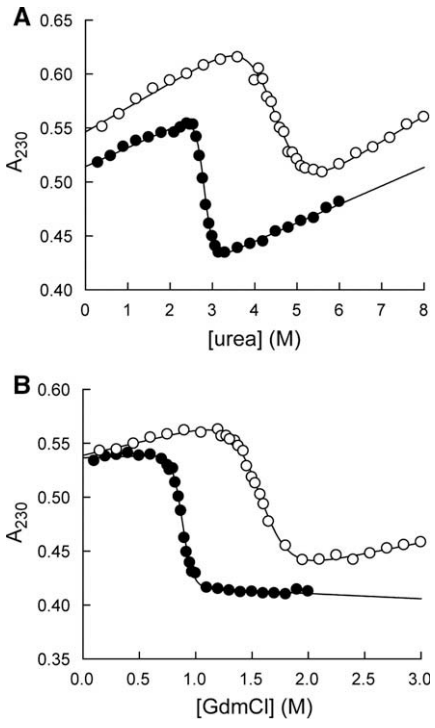

Fig. 2. Determination of the thermodynamic stabilities of MBP and RNase H monitored by A_{230}. Equilibrium unfolding of MBP (●) and RNase H (○) in urea (A) and GdmCl (B) was monitored by A_{230}. A_{230} of each sample was determined in a quartz cuvette. The C_m and m values determined by fitting the absorbance to Eq. (1) are reported in Table 1.

Determination of equilibrium unfolding of MBP by A_{230} on 96-well titer plate

Currently, it is not possible to measure CD signals of protein samples in microtiter plates. UV absorbance, however, can be measured in microtiter plates, which may allow determination of A_{230} of many samples simultaneously. By using A_{230} as a probe, we have explored the possibility of determining protein stability in 96-well microtiter plates. One hundred-microliter aliquots of 50 μg/mL MBP was prepared in a buffer containing varying concentrations of urea. After overnight incubation, A_{230} of each aliquot was determined simultaneously in a UV-transparent 96-well microtiter plate. Because the path length of the solution in the microtiter plate is not 1 cm, the A_{230} measured in the microtiter plate was dif-

Table 1
Monitoring equilibrium unfolding of MBP and RNase H by A_{230}.

	Denaturant	Methods	C_m (M)	m (kcal/mol M)	ΔG_{unf}° (kcal/mol)
MBP	Urea	CD[a]	2.83 ± 0.01	5.2 ± 0.3	14.7 ± 0.9
		A_{230} (cuvette)	2.81 ± 0.01	5.7 ± 0.3	16.0 ± 0.8
		A_{230} (multititer)	2.76 ± 0.01	4.2 ± 0.3	11.6 ± 0.8
	GdmCl	A_{230} (cuvette)	0.88 ± 0.00$_3$	13.1 ± 0.8	11.5 ± 0.7
		A_{230} (multititer)	0.87 ± 0.01	12.2 ± 1.3	10.6 ± 1.1
RNase H	Urea	CD[b]	4.62 ± 0.01	2.1 ± 0.1	9.7 ± 0.5
		A_{230} (cuvette)	4.47 ± 0.03	1.7 ± 0.1	7.6 ± 0.5
	GdmCl	A_{230} (cuvette)	1.57 ± 0.01	4.8 ± 0.2	7.5 ± 0.3

The C_m and m values (±SE) were determined by a nonlinear regression with Eq. (1). ΔG_{unf}° values were determined by multiplying C_m and m values.
[a] From [13].
[b] From [11].

ferent from the value measured in a 1-cm cuvette. To compare with the cuvette result, the fraction of folded protein (f_{fold}) in each concentration of urea was calculated after the curve-fitting of A_{230} to Eq. (1). The results by a cuvette and a 96-well titer plate are very similar (Fig. 3A). Though the microtiter plate result is somewhat noisier than the cuvette result, consistent C_m values were determined by the two methods (2.81 ± 0.01 M in a cuvette and 2.76 ± 0.01 M in a microtiter plate) (Table 1). Equilibrium unfolding of MBP in GdmCl was also monitored in a 96-well microtiter plate by using A_{230} (Fig. 3B). The results again demonstrate good agreement with the cuvette results. The C_m values of MBP in GdmCl determined in a cuvette and a microtiter plate are $0.88 \pm 0.00_3$ and 0.87 ± 0.01 M, respectively. However, m values determined in the microtiter plate show greater statistical uncertainty than the values determined in a cuvette.

Determination of unfolding kinetics by A_{230}

We also tested A_{230} as a probe to investigate unfolding kinetics of MBP and RNase H. To determine unfolding rate constants and kinetic m values, we monitored unfolding of MBP by A_{230} at nine different concentrations of urea. Fig. 4A shows the change in A_{230} of MBP on unfolding in 5.85 M urea. A_{230} decreased from 0.55 to 0.40 exponentially, which was fit to a first-order rate equation perfectly. It appears that the signal-to-noise ratio of kinetics data by A_{230} is as good as typical kinetics measurements by CD. Unfolding at other concentrations of urea also shows single-exponential decay from 0.55 to 0.40. In these kinetic experiments, the absorbance was zeroed with the buffer containing urea before protein was added. Therefore, the contribution of urea to the absorbance was eliminated. The consistent initial and final absorbance values in different concentrations of urea also corroborate that the baseline changes observed in equilibrium unfolding in Fig. 3A result mostly from the absorbance of urea.

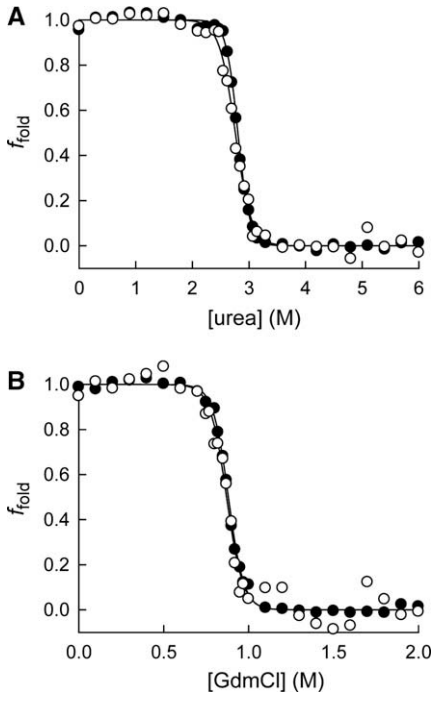

Fig. 3. Determination of the thermodynamic stability of MBP in equilibrium unfolding of MBP monitored by a 96-well plate reader. Equilibrium unfolding of MBP in urea (A) and GdmCl (B) was monitored in a 96-well multititer plate (○). The A_{230} was converted to fraction of folded proteins (f_{fold}). Fraction of folded proteins determined in a quartz cuvette is shown for comparison (●). The C_m and m values determined by fitting the absorbance to Eq. (1) are reported in Table 1.

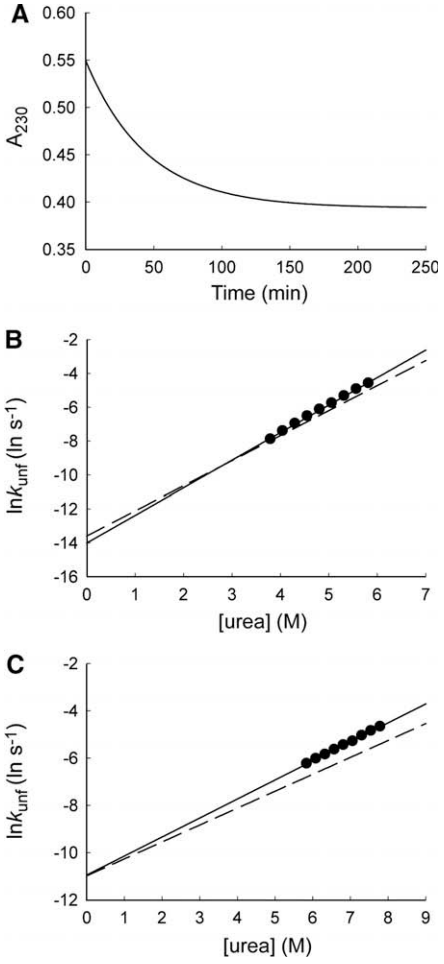

Fig. 4. Determination of unfolding kinetics of MBP and RNase H by A_{230}. (A) The exponential decay of A_{230} on unfolding of MBP in 5.85 M urea. (B and C) The log values of k_{unf} for MBP (B) and RNase H (C) unfolding are plotted against urea concentration. The $k_u(H_2O)$ values and m_u were determined by fitting the plot to Eq. (2). Unfolding kinetics data from CD measurement (- - -) are shown for comparison [11,14].

The plot of the natural logarithm of k_u versus the concentration of urea shows a linear correlation as expected (Fig. 4B). By extrapolating the plot to 0 M urea, $k_u(H_2O)$ was determined to be 8.22×10^{-7} s^{-1}. The kinetic m value of MBP unfolding was also determined to be 0.96 ± 0.01 kcal/mol M (Table 2). Fig. 4B also shows the unfolding kinetics data determined by CD [14], which overlap well with those determined by A_{230}. The $k_u(H_2O)$ and kinetic m values of MBP unfolding determined by the two different methods are consistent (Table 2).

We also investigated unfolding kinetics of RNase H by A_{230}. The natural logarithm of k_u of RNase H also shows a good linear correlation with urea concentration (Fig. 4C). The kinetic m value was

Table 2
Determination of unfolding kinetic constants of MBP and RNase H in urea by A_{230}.

	Method	$k_u(H_2O)$ (s^{-1})	m_u (kcal/(mol M))
MBP	CD[a]	$(12.4 \pm 1.1) \times 10^{-7}$	0.87 ± 0.01
	A_{230}	$(8.2 \pm 1.1) \times 10^{-7}$	0.96 ± 0.01
RNase H	CD[b]	$(1.69 \pm 0.04) \times 10^{-5}$	0.42 ± 0.02
	A_{230}	$(1.75 \pm 0.11) \times 10^{-5}$	0.47 ± 0.01

$k_u(H_2O)$ and m_u values (±SE) were determined by nonlinear regression with Eq. (2).
[a] From [14].
[b] From [11].

determined to be 0.47 ± 0.01 kcal/(mol M), which is in good agreement with that determined by CD (0.42 ± 0.02 kcal/(mol M) [11]) (Table 2). $k_u(H_2O)$ determined by A_{230} (1.75×10^{-5} s^{-1}) shows excellent agreement with that determined by CD (1.69×10^{-5} s^{-1} [11]). The consistency of the parameters determined by CD and A_{230} suggests that A_{230} is a valid probe to monitor global unfolding of these proteins.

Discussion

The chromophores for UV absorbance in proteins are divided into two classes: peptide bonds and aromatic side chains [3]. Absorbance at 280 nm by aromatic side chains has been commonly used to determine protein concentrations and also to monitor conformational changes in proteins. Absorbance of amide bonds near 190 nm has significant sensitivity to conformational changes [15]. However, the use of 190 nm is not practical due to strong influence from buffer and salt components. A_{230} is a relatively unknown probe in the UV region. The physical origin of the change in A_{230} has been suggested to be identical with the event causing the change in A_{280}. The change in A_{230} on protein unfolding always correlates well with the change in A_{280} [6–8]. Apparently, when aromatic amino acids experience changes in their environment, their absorptions at both wavelengths change simultaneously.

It has been shown that tryptophan contributes most to the change in A_{230} [8,9]. Tyrosine and phenylalanine residues also absorb at 230 nm but their contributions are much less significant than tryptophan residues. MBP and RNase H have eight and six tryptophan residues, and both proteins show considerable changes in A_{230} on unfolding (Fig. 1). The protein concentration used for the thermodynamic and kinetic studies here was only 50 μg/mL, which is comparable with the protein concentration typically used for CD experiments. A_{280} is not sensitive enough to allow monitoring of protein unfolding with this low concentration of proteins. The required protein concentration for A_{230} is dependent on the content of tryptophan residues. If a protein has only a few tryptophan residues, higher concentrations of protein may be required.

Frequently protein unfolding is monitored with multiple probes to unravel the presence or the absence of intermediates [16]. It is quite common for a single spectroscopic probe to fail to detect an intermediate because the intermediate possesses a spectroscopic property similar to either native or unfolded proteins. When multiple probes report noncoincidental transitions, the existence of intermediates is suggested and the nature of the transitions can be further investigated [17,18]. Therefore, it is quite beneficial to have probes sensitive to different aspects of conformational changes on unfolding. The physical origin of the change in A_{230} is clearly different from far UV CD that is sensitive to the changes in the secondary structures of proteins. Because 230 nm is within the range of typical CD experiments and instrumentation of CD is based on UV absorption of polarized light, simultaneous monitoring of CD and A_{230} by a spectropolarimeter seems to be feasible without an instrumental modification. Information from both probes would be quite useful in elucidating the presence of intermediates that is not detected by a single probe.

Rapid determination of the thermodynamic stability of proteins has many useful applications in protein engineering and drug discovery. Recently, several approaches have been developed to monitor protein unfolding in high-throughput format. Binding of hydrophobic fluorescent dyes to thermally denatured proteins has been exploited to monitor thermal unfolding of proteins as well as the stabilization of proteins on ligand binding for drug screen purposes [19]. Mass spectrometric monitoring of the exchange of amide backbone hydrogen with solvent deuterium, known as "SUPREX," is also another successful development of a high-throughput analysis of protein stability [20]. "Pulse proteolysis" measures the change in proteolytic susceptibility of proteins on unfolding, which can be used to determine C_m values quantitatively without using spectroscopic methods [10]. Measurement of A_{230} shows the possibility of monitoring protein stabilities in a high-throughput fashion in 96-well microtiter plates (Fig. 3). CD spectroscopy in a 96-well format is not yet available. Tryptophan fluorescence has been used to monitor protein unfolding in 96-well titer plates [21]. Our results demonstrate that A_{230} is also a useful alternative to fluorescence for high-throughput approaches. Availability of cost-effective disposable UV transparent microtiter plates makes this application of A_{230} even more appealing.

Though the measurement of A_{230} in microtiter plates is somewhat noisy, C_m values were determined quite reliably. Accurate determination of m values is not guaranteed with this approach (Table 1), as it is well known that m values are much more difficult to determine accurately than C_m's. Because it is possible to estimate m values from the size of proteins using statistical data, ΔG_{unf} values still can be determined with only C_m values as in the applications of SUPREX and pulse proteolysis [10,20]. Moreover, when protein stability is determined with different ligands for drug screening, the change in C_m alone provides enough information to monitor ligand binding. Considering the ease of instrumentation for the measurement, A_{230} is a useful and versatile tool for biophysical investigation of protein energetics and for biotechnological applications of stability measurement.

Acknowledgments

We thank Jonathan Schlebach and Youngil Chang for helpful comments on the manuscript.

References

[1] R.F. Greene, C.N. Pace, Urea and guanidine-hydrochloride denaturation of ribonuclease, lysozyme, α-chymotrypsin, and β-lactoglobulin, J. Biol. Chem. 249 (1974) 5388–5393.
[2] G.R. Grimsley, B.M.P. Huyghues-Despointes, C.N. Pace, J.M. Scholtz, in: R.J. Simpson (Ed.), Purifying Proteins for Proteomics: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2004, pp. 535–566.
[3] R.C. Cantor, R.P. Shchimmel, Biophysical Chemistry, Freeman, San Francisco, 1980.
[4] A.N. Glazer, E.L. Smith, Studies on the ultraviolet difference spectra of proteins and polypeptides, J. Biol. Chem. 236 (1961) 2942–2947.
[5] A.N. Glazer, E.L. Smith, Effect of denaturation on the ultraviolet absorption spectra of proteins, J. Biol. Chem. 235 (1960) PC43–PC44.
[6] C.J. Martin, G.M. Bhatnagar, Unfolding reactions of proteins. I. Kinetic and equilibrium measurements of diisopropylphosphorylchymotrypsin and chymotrypsinogen in urea, Biochemistry 5 (1966) 1230–1241.
[7] D.S. Eisenberg, J.T. Edsall, Spectrophotometric titrations of human serum albumin and reduced carboxymethylated albumin, Science 142 (1963) 50–51.
[8] S. Mora, P. Elodi, Investigation of the near and far ultraviolet denaturation difference spectra of dehydrogenases, Eur. J. Biochem. 5 (1968) 574–582.
[9] J.W. Donovan, Changes in ultraviolet absorption produced by alteration of protein conformation, J. Biol. Chem. 244 (1969) 1961–1967.
[10] C. Park, S. Marqusee, Pulse proteolysis: a simple method for quantitative determination of protein stability and ligand binding, Nat. Methods 2 (2005) 207–212.
[11] T.M. Raschke, J. Kho, S. Marqusee, Confirmation of the hierarchical folding of RNase H: a protein engineering study, Nat. Struct. Biol. 6 (1999) 825–831.
[12] C.N. Pace, F. Vajdos, L. Fee, G. Grimsley, T. Gray, How to measure and predict the molar absorption coefficient of a protein, Protein Sci. 4 (1995) 2411–2423.
[13] C. Park, S. Zhou, J. Gilmore, S. Marqusee, Energetics-based protein profiling on a proteomic scale: identification of proteins resistant to proteolysis, J. Mol. Biol. 368 (2007) 1426–1437.
[14] Y.-R. Na, C. Park, Investigating protein unfolding kinetics by pulse proteolysis, Protein Sci. 18 (2009) 268–276.
[15] K. Rosenheck, P. Doty, The far ultraviolet absorption spectra of polypeptide and protein solutions and their dependence on conformation, Proc. Natl. Acad. Sci. USA 47 (1961) 1775–1785.
[16] P.S. Kim, R.L. Baldwin, Specific intermediates in the folding reactions of small proteins and the mechanism of protein folding, Annu. Rev. Biochem. 51 (1982) 459–489.
[17] B. Robson, R.H. Pain, The mechanism of folding of globular proteins: equilibria and kinetics of conformational transitions of penicillinase from *Staphylococcus*

aureus involving a state of intermediate conformation, Biochem. J. 155 (1976) 331–344.
[18] S. Tandon, P.M. Horowitz, The detection of kinetic intermediate(s) during refolding of rhodanese, J. Biol. Chem. 265 (1990) 5967–5970.
[19] M.D. Cummings, M.A. Farnum, M.I. Nelen, Universal screening methods and applications of ThermoFluor, J. Biomol. Screen. 11 (2006) 854–863.
[20] S. Ghaemmaghami, M.C. Fitzgerald, T.G. Oas, A quantitative, high-throughput screen for protein stability, Proc. Natl. Acad. Sci. USA 97 (2000) 8296–8301.
[21] J.P. Aucamp, A.M. Cosme, G.J. Lye, P.A. Dalby, High-throughput measurement of protein stability in microtiter plates, Biotechnol. Bioeng. 89 (2005) 599–607.