Proteolysis as a measure of the free energy difference between cytochrome *c* and its derivatives

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Abstract

Limited cleavage of oxidized and reduced horse heart cytochrome *c* (Cyt *c*) and the azide complex of Cyt *c* by proteinase K at room temperature yields a single cut within the central loop (36–60 in the sequence). Using an assay that allows spectroscopic evaluation of the fraction of intact protein as a function of time, together with a simple kinetic model for proteolysis, fluctuation opening of the loop can be related to the free energy of the corresponding protein. This allows us to estimate quantitatively the free energy difference between the oxidized form of Cyt *c* and other states using proteolysis as a probe. The results we obtain indicate that oxidized Cyt *c* is 2.0 kcal mol⁻¹ less stable than the reduced form, and 0.07 kcal mol⁻¹ is more stable than the Cyt *c*: azide complex at 25 °C. These values agree in magnitude with results from hydrogen exchange and unfolding studies, suggesting that the stability of a protein can be directly related to its structural dynamics.

Keywords: circular dichroism; cytochrome *c*; free energy; proteinase K; proteolysis

Limited proteolytic digestion has been applied to investigate the conformational stability of proteins for many years (Fontana et al., 1986; Johnson & Price, 1986; Novotny & Broccoleri, 1987; Ellison et al., 1995). Use of the method is predicated on the assumptions that the proteolysis event is dictated by the stereochemistry and accessibility of the protein substrate (Fontana et al., 1997) as well as by the activity and specificity of the attacking enzyme. For example, while trypsin tends to cleave native and unfolded proteins, chymotrypsin is much more active against unfolded proteins, reflecting increased exposure of aromatic side chains in unfolded proteins. Studies of initial attack by thermolysin reveals that the initial sites of attack by this enzyme occur in regions of flexibility of the substrate (Fontana et al., 1986; Cohen et al., 1995). In a previous study, we used the enzyme proteinase K to monitor thermal unfolding of horse heart Cyt *c* (Wang et al., 1998). Exposure of Cyt *c* at room temperature or on ice to proteinase K produces two bands on SDS-PAGE, which result from cutting at residues 48–49 and 50–51 (Fig. 1) located in the center loop of Cyt *c* (Wang et al., 1998). Similar results are obtained using reduced Cyt *c* and the Cyt *c*:azide complex (Fig. 2). Proteinase K was selected because it is an extremely stable enzyme with broad substrate specificity. Since the substrates of this study have the same sequence, any differences in cleavage rates should reflect changes in the stability or dynamics of these proteins. We suggest that in

such cases limited proteolysis can be used to estimate free energy differences between these states quantitatively.

In general, equilibrium unfolding experiments offer the simplest approach to determining thermodynamic aspects of protein stability (Pace, 1975; Pace & Scholtz, 1996). Spectroscopic measurements of proteins as a function of added denaturant or temperature allow determining the van't Hoff thermodynamic parameters for the N-U transition process, while calorimetry makes it possible to measure complete transition profiles without recourse to two state transition models (Yu et al., 1994). In the case of Cyt *c*, spectroscopic measurements reveal that the unfolding mechanism of all states of horse heart Cyt *c* appears to be complex (Ikai et al., 1973; Tsong, 1973; Wang et al., 1998) in that intermediate states are present. It is thus difficult to determine ΔG° values using direct methods. Recently a detailed analysis of the equilibrium unfolding pathway in Cyt *c* was based on hydrogen exchange rate measurements as a function of added denaturing solvent (Bai et al., 1995). These experiments clearly reveal a sequential opening process; however, the mechanism of the fluctuations leading to exchange of backbone NH protons remains unknown, and the results are thus indirect.

In this article, we propose a simple theoretical model to study the free energy difference between Cyt *c* and its derivatives. This model links the proteolytic liability to fluctuations in the native state of the protein. CD measurements under mildly unfolding conditions are used to monitor the fraction of uncut protein in the reaction mixture (see Fig. 3). Applying this assay to Cyt *c* under different conditions, we find that oxidized Cyt *c* is 2.0 kcal mol⁻¹ less stable than the reduced form, and 0.07 kcal mol⁻¹ more stable

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than Cyt *c*: azide complex. These values are in rough agreement with results from van't Hoff unfolding data (Pfeil, 1981; Nall, 1996) and the hydrogen exchange analysis (Nabedryk-Viala et al., 1976; S.W. Englander, unpubl. data). They arguably support the idea that local unfolding of the target site mediates scission by PK, and that the stability of the protein influences the dynamics of loop fluctuations. Thus subdomain protein fluctuations may be functionally relevant.

The specific activity of proteinase K (PK) enzyme preparation in our experiments is comparable to that described by Bajorath et al. (1988) in their study of the temperature optimum of the enzyme.

Fig. 3. The strategy to measure the quantity of uncleaved protein in the reaction mixture. Mild denaturation is applied to the proteolysis mixture to make cleaved protein unfold while the uncleaved protein still keeps its secondary structure. In this condition CD can be used to measure the quantity of uncleaved protein substrate by comparing with standard sample.

Fig. 2. The SDS-PAGE analysis of Cyt *c* and its derivatives digested with proteinase K at room temperature. The high resolution Tricine buffer system SDS-PAGE (Schaegegg & von Jagow, 1987) was used and the gel was stained with Coomassie Blue. The enzyme/protein ratio is 1:50 (w/w), the reaction was stopped by PMSF. Line (1) protein markers, (2) proteinase K control, (3) Cyt *c* control, (4) Cyt *c*, 1 h reaction, (5) Cyt *c*:azide complex, 1 h reaction, (6) reduced Cyt *c*, 2 h reaction.

Fig. 4. The activity of proteinase K in different buffers. The reaction was taken at room temperature for 1 h as described under Materials and methods. The buffers are 20 mM Tris/Hcl pH = 7.5 containing 20 mM sodium chloride (circle), sodium azide (triangle), or sodium ascorbic acid (square).

Table 1. The apparent digestion speeds (unit: $\mu\text{M min}^{-1}$) obtained from different detection conditions: Reaction mixture in 1.5 M guanidine or at different temperatures without guanidine

Detection condition	1.5 M Guanidine at			
	25 °C	25 °C	50 °C	55 °C
Azide complex	2.13	—	—	—
Oxidized form	1.55	1.26	1.21	1.26
Reduced form	0.043	—	—	—

the CD spectrometer is estimated to be 3%. By comparing the proteolysis kinetics of oxidized Cyt *c* under different measurement conditions, it appears that the rate measured in GdmCl is slightly

higher than that measured by unfolding at high temperature. The discrepancy can be traced to retention of ellipticity at 222 nm in the thermally unfolded fragments (Sosnick et al., 1997). This led us to select GdmCl denaturation as a reference for monitoring the extent of cleavage.

By comparing the proteolysis rates (Table 1), we find that Cyt *c*:azide is $0.07 \text{ kcal mol}^{-1}$ less stable than oxidized Cyt *c* while reduced Cyt *c* is $2.0 \text{ kcal mol}^{-1}$ more stable. These data agree roughly with the values obtained by the Englander group: in their HX exchange model, the energy difference between Cyt *c* and reduced Cyt *c*, for the N- and C-terminal helices, is 6 kcal mol^{-1} . For other subdomains the difference is less, about $3.2 \text{ kcal mol}^{-1}$ (S.W. Englander, unpubl. results; related article Bai et al., 1995). Because the initial attacking sites of proteinase K in these reaction are located in the 50's loop of Cyt *c*, it is more likely that our results reflect the free energy difference of the loop fluctuations and not the protein as a whole, represented by the N- and C-helical region (Bai et al., 1995).

Fig. 5. The kinetic proteolysis of Cyt *c* measured by CD at 222 nm. The values were normalized with the signal at the beginning of the reaction. All the digestion products were diluted to a final buffer condition 20 mM Tris-HCl (pH = 7.5) containing 20 mM NaCl and 1.5 M GdmCl at room temperature when the CD measurements were performed: (A) azide complex, (B) oxidized form, (C) reduced form.

Fig. 6. The kinetic proteolysis of oxidized Cyt *c* measured by CD at 222 nm. The digestion products were diluted to a final buffer condition 20 mM Tris-HCl (pH = 7.5) containing 20 mM NaCl. The measurements were performed at different temperatures and all the values were normalized using the CD value measured at 25 °C at the beginning of the reaction: 25 °C (empty square), 50 °C (empty circle), and 55 °C (black circle).

The existence of structural subdomains of Cyt *c* with free energies below that of the N- and C-terminal α -helical interaction has been confirmed in a model peptide study (Wu et al., 1993). Here we find that proteolysis of Cyt *c* and its derivatives with proteinase K results exclusively in cleavage of the same site in their central loops. Relating the cutting to the stability of the loop in each case gives results in agreement with this model peptide study. It is also known that the covalently attached heme group is essential for Cyt *c* folding (Stellwagen & Rysavy, 1972; Fisher et al., 1973). The faster fluctuation of the central loop leads to higher exposure of the ferric heme environment to the solvent in agreement with the result of a crystal structural study (Takano & Dickerson, 1982). Our data further suggest that the heme group stabilizes Cyt *c* by interacting with its central loop and lets Cyt *c* form a loop-heme subdomain together with an N- and C-terminal helical interaction domain. The stronger the heme-loop interaction, the more stable the whole protein. Conversely, the loop is more accessible to proteolysis in the oxidized protein, and this may influence the lifetime of Cyt *c* in vivo.

Materials and methods

Materials

Horse heart Cyt *c* was purchased from Sigma Chemical Company (St. Louis, Missouri) and was used without further purification. Proteinase K was obtained from USB (Cleveland, Ohio). Suc-(Ala)₃-pNA was purchased from Serva (Germany). Guanidinium chloride (GdmCl), Tris base, sodium chloride, sodium azide, and sodium ascorbate were all from Sigma Chemical Company, highest purity grades. Azide:Cyt *c* complex was obtained by mixing Cyt *c* with a 20-fold excess of sodium azide (final concentration 20 mM) in Tris-HCl buffer at pH 7.5. Reduced Cyt *c* was got by

putting Cyt *c* in 20 mM sodium ascorbic acid in Tris-HCl buffer at pH = 7.5 (Viola et al., 1996).

Limited protease digestion

Digestion experiments were carried out with a protein:proteinase (w/w) ratio of 50:1 at room temperature in 20 mM Tris-HCl buffer pH = 7.5 containing 20 mM sodium chloride, sodium ascorbic acid, or sodium azide. The experiments were carried out with 3.8 mg/mL ($3 \times 10^2 \mu\text{M}$) Cyt *c* and 76 $\mu\text{g/mL}$ (2.6 μM) proteinase K. The reaction was stopped by adding PMSF.

CD data collecting

Experiments were designed to monitor the undigested protein using circular dichroism measurements with an AVIV 60DS instrument. To make the result more accurate, data were collected in the kinetic mode, and the final data were an average of the complete data set recorded. The collecting time was 3 min and 1 s per signal point was used in each case. The digested samples were diluted to a final buffer condition: 1.5 M GdmCl containing 20 mM Tris-HCl, 20 mM NaCl, pH = 7.5.

Activity of proteinase K in different buffers

The activity of proteinase K was calibrated using the peptide substrate Suc-(Ala)₃-pNA. The assays were prepared in 20 mM Tris/HCl (pH = 7.5) containing 20 mM sodium chloride, sodium azide, or sodium ascorbic acid with a final concentration of 5 $\mu\text{g/mL}$ proteinase K, and the substrate concentrations from 0.1 mM to 2.0 mM. Assays of 1 mL volume were performed at room temperature for 1 h, and the reaction was stopped by addition of 0.2 mL glacial acetic acid. The absorbance of the liberated nitro-aniline was measured at 410 nm using 1 cm path length cell on Perkin Elmer 552 spectrophotometer (Bajorath et al., 1988).

Fluctuation model

If proteases initially attack flexible segments of protein substrates, then we assume these regions are exposed in some kind of closed to open reaction, similar to the original Linderstrom-Lang scheme for hydrogen exchange (Englander & Kallenbach, 1984). The reaction is indicated in Equation 1

In this scheme, the structural opening reaction enters the rate expression as an equilibrium step. The observed cutting rate k_{ex} is then determined by the enzyme cutting rate in the fully open state k_{cut} multiplied by the equilibrium opening constant K_{op} (Equation 2):

$$k_{\text{ex}} = K_{\text{op}} k_{\text{cut}}. \quad (2)$$

The free energy of the opening reaction ΔG_{op} can then be calculated by Equation 3,

$$\Delta G_{\text{op}} = -RT \ln K_{\text{op}} = -RT \ln(k_{\text{ex}}/k_{\text{cut}}) \quad (3)$$

where R is the gas constant and T is the absolute temperature. For the case of Cyt c and its derivatives, if the same proteolysis condition is applied, the k_{cut} rates will be same, so the free energy difference of opening reactions $\delta\Delta G_{\text{op}}$ in the various states can be determined (Equation 4):

$$\delta\Delta G_{\text{op}}(1,2) = \Delta G_{\text{op}}(2) - \Delta G_{\text{op}}(1) = -RT \ln(k_{\text{ex}2}/k_{\text{ex}1}). \quad (4)$$

Thus free energy differences in the opening reaction should reflect the differential stabilization of the target region among the native states.

Assay of proteolysis rates

The secondary structures, α -helix, β -sheet, and coil, have significant circular dichroism signals in far UV region (Greenfield et al., 1967). At 222 nm the signal mainly reflects the content of helical structure in the sample. The concentration of intact Cyt c during proteolysis, whose only regular secondary structure is helix, can be detected using CD measurement by comparing with Cyt c samples with known concentration.

At room temperature the PK digestion leads to cutting within the central loop of Cyt c (Wang et al., 1998). The resulting fragmented structure resists further digestion implying retention of native-like structure, in agreement with reconstruction experiments performed on Cyt c by Hantgan and Taniuchi (1978). This makes it difficult to determine the concentration of intact Cyt c molecules. However, the fragmented protein is thermodynamically less stable at low concentrations than the intact Cyt c , allowing us to discriminate intact Cyt c from the severed fragments by placing the digested sample in moderate denaturant conditions, where the intact Cyt c retains its folded structure while the cleaved products unfold. This situation is schematically illustrated in Figure 3.

The concentration of intact protein is calculated by Equation 5:

$$C(t) = C_0[\theta]/[\theta]_0. \quad (5)$$

With the concentrations of remaining intact protein at different digestion time, the cutting rate v can be written as Equation 6:

$$v = dC/dt. \quad (6)$$

When the digestion time is relatively short, it can be written as Equation 7:

$$C(t) \approx C_0 - vt \approx C_0 - C_0 k_{\text{ex}} t. \quad (7)$$

From the $[C] \sim t$ diagram, the linear slope is $v = k_{\text{ex}} C \approx k_{\text{ex}} C_0$.

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