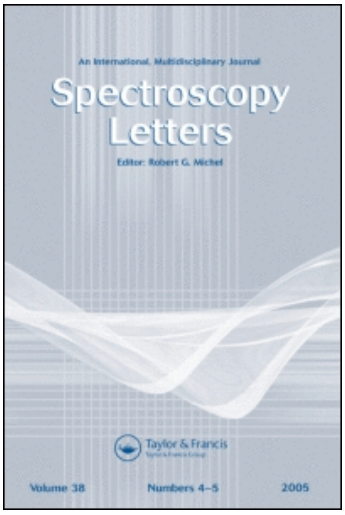

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The Detection of Unfolding Intermediates of Soybean Lipoxygenase-1 during Urea Denaturation by Fluorescence Spectroscopy

Ying Wu[a]; Zhi-Xin Wang[a]
[a] National Laboratory of Biomacromolecules, Institute of Biophysics, P. R. China

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The Detection of Unfolding Intermediates of Soybean Lipoxygenase-1 during Urea Denaturation by Fluorescence Spectroscopy

Key words: Fluorescence spectroscopy; unfolding intermediates; enzyme inactivation; protein denaturation

Ying Wu, Zhi-Xin Wang

National Laboratory of Biomacromolecules
Institute of Biophysics, Academia Sinica
Beijing 100101, P. R. China

ABSTRACT

The unfolding of soybean lipoxygenase-1 during urea denaturation has been followed by activity assays and fluorescence measurement. The presence of stable intermediates during unfolding for both ferrous and ferric forms of lipoxygenase-1 were observed. In the presence of 6.0 M urea, the unfolding of soybean lipoxygenase-1, as monitored by fluorescence intensity, is a triphasic process, while the inactivation of the enzyme shows a single-phase kinetics. The rate constant of

inactivation is consistent with that of the fast conformational change of the enzyme. Based on these, a minimal scheme containing two intermediates was proposed to interpret the unfolding of lipoxygenase-1 induced by urea.

INTRODUCTION

Although all the information necessary for the formation of the three-dimensional structure in protein is contained in the amino acid sequence, the folding mechanism of globular proteins is still not fully understood [1-5]. A variety of equilibrium and kinetic techniques have been applied to the study of the folding/unfolding of a number of globular proteins. Since the transition state for unfolding is generally judged to be closer to native state than to unfolded state, studying unfolding intermediates should be extremely helpful in learning whether there is a unique transition state and, if so, in determining its properties [6]. The basic test used in the past to probe for unfolding intermediate was to compare the kinetics of unfolding monitored by various probes to ask if the unfolding curves monitored by different probes are superimposable [7]. For small globular proteins, a simple two-state model can be used to analyze the unfolding transition. In the case of larger multidomain proteins, the presence of stable intermediates has been observed during unfolding [3,8].

Soybean lipoxygenase-1 (EC 1.13.11.12) catalyzes the dioxygenation of linoleic acid to form 13-hydroperoxy-9,11-octadecadienonic acid (HPOD) [9,10]. The enzyme is a single polypeptide protein of molecular weight 100,000, containing 1 mol of iron cofactor per mole of enzyme [11-13]. During the normal course of catalysis, the Fe^{2+} cofactor is converted into the Fe^{3+} form by the action of the reaction product HPOD, and the latter Fe^{3+} form is maintained during the steady-state turnover of the enzyme [14]. The transition in the oxidation state of the iron

cofactor is marked by changes in the spectroscopic properties of both the protein and cofactor species. These include (1) the appearance of an EPR signal at $g = 6.1$ due to the transition of the high-spin Fe^{2+} to the high spin Fe^{3+}, (2) about a 30% decrease in the tryptophan fluorescence, and (3) the appearance of an absorption band at 330 nm [15-19]. The structure of lipoxygenase-1 resolved to 2.6 Å resolution shows that the enzyme has two domains [20], and therefore it is an ideal candidate for folding studies of multi-domain enzymes which require essential co-factors for the catalytic activity. In the present study, the unfolding of soybean lipoxygenase-1 monitored by fluorescence suggesting the presence of stable intermediates during unfolding.

MATERIALS AND METHODS

Materials

Soybean lipoxygenase (type I-B) and linoleic acid (highest purity grade, purity higher than 99%) were obtained from Sigma Chemical Co. Urea (ultra-pure) was from Boehringer-Mannheim, and solutions were always freshly prepared from a recrystallized sample [21]. Other chemicals were local products of analytical grade used without further purification. Centricon-10 microconcentrators was purchased from Amicon. Sodium borate buffer (0.1M, pH 10.0) was used in all experiments unless stated otherwise. All solutions (including buffer) were prepared in Milli-Q water.

Methods

Soybean lipoxygenase activity was routinely assayed by monitoring the increase in absorbance at 234nm due to formation of HPOD (ε_{max}=25,000$M^{-1}cm^{-1}$) using a Shimadzu UV-250 spectrophotometer thermostated at 20°C. For

determination of specific activities, the enzyme activity was measured at 25°C in a 0.2M sodium borate buffer, pH9.0, containing 23mg/L Tween 20, utilizing 83μM linoleic acid as substrate as described previously by Axelrod et al. [22]. Protein concentration was determined by measuring the absorbance at 280nm ($E_{280}^{1\%} = 14.0$; [22]).

The commercial preparation of soybean lipoxygenase was further purified by chromatography on Mono Q-FPLC column following the method of Wang [23] with a additional step of purification on a Superose 12-FPLC column. The purified lipoxygenase-1 showed a single protein band when subjected to SDS-PAGE analysis and had a specific activity of 160 units/mg under the standard assay conditions[22].

To avoid oxidation of linoleic acid by atmospheric oxygen, the vial of the highest purity grade linoleic acid (>99% pure) was broken in an anaerobic chamber (flushed with high purity nitrogen). The stock aqueous solution of linoleic acid was freshly prepared in 0.1 M borate buffer pH 10.0. The Fe^{3+} form of enzyme was freshly prepared by addition of a 2–3-fold molar excess of linoleic acid to the native enzyme at pH 10.0 and 0°C and stored at this temperature during the course of entire experiment.

Fluorescence Measurements

Equilibrium unfolding as a function of urea concentration was monitored by fluorescence spectroscopy by exciting the tryptophans at 280 nm on a Hitachi F-4010 fluorimeter. All samples were allowed to equilibrate fully at the appropriate final urea concentration at 20°C before spectra were taken.

Kinetics of unfolding was also followed by fluorescence spectroscopy. The reaction mixture was continuously stirred by a magnetic stirrer installed at the bottom of the cuvette holder. This stirring device allowed us to start reaction and data collection (by computer) within 8 seconds after initiating the reaction.

Fluorescence measurements were made by exciting at 280 nm. The emission intensity was monitored at wavelengths 332nm. Slit widths of 5nm and 10nm were used for excitation and emission, respectively.

Computer fitting of unfolding kinetic data

Nonlinear least-squares fits to kinetic data of unfolding were obtained by using a computer program and the equation:

$$F(t) = \sum_i F_i \exp(-k_i t) + F_\infty \qquad (1)$$

where $F(t)$ is the emission intensity at time t, F_∞ is final emission intensity at time infinity, F_i is the amplitude corresponding to the individual phase, i, at zero time, and k_i is the associated rate constant.

RESULTS AND DISCUSSION

Changes in activity and intrinsic fluorescence of Lipoxygenase-1 in different concentrations of urea solutions

To determine the degree of unfolding of soybean lipoxygenase-1 by urea, the ferrous enzyme was incubated in various concentrations of urea in 0.1M borate buffer, pH 10.0 at 20°C for 12 hours; 10 μl aliquots of sample were removed for assay, with the same concentration of urea present in the assay mixture as in the original incubation. The extents of inactivation in urea solutions are shown in Figure 1. It can be seen from this figure that there was a progressive loss of enzyme activity. The enzyme is completely inactivated by incubation with 4.0 M urea for 12 hours, whereas control samples without urea show complete activity.

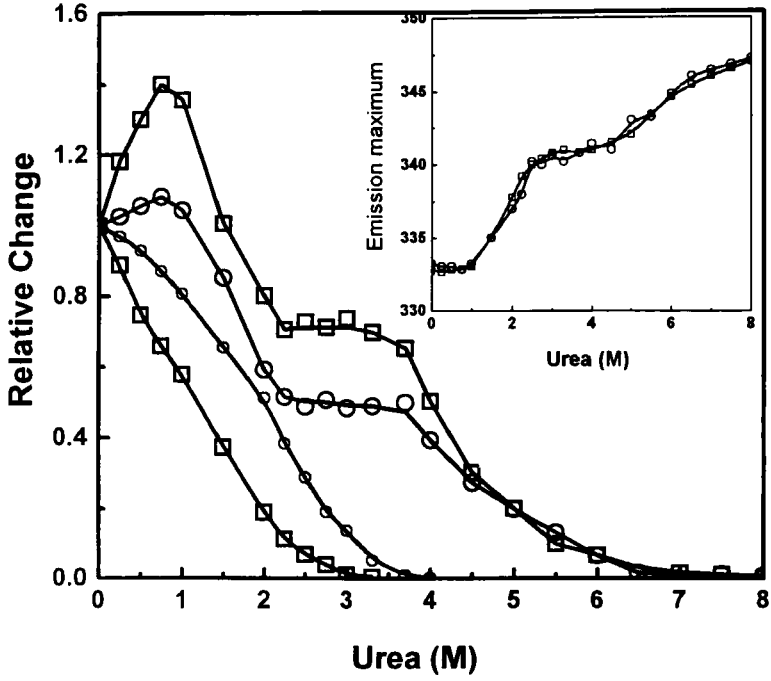


Figure 1. Comparison of activity and conformational changes in soybean lipoxygenase-1 at different concentrations of urea solution at pH 10.0, 20°C. The filled symbols refer to relative emission intensity at 332 nm, with an excitation wavelength of 280 nm, for the ferrous (O) and ferric enzyme (□). The open symbols refer to relative activity for the ferrous (O) and ferric enzyme (□). The enzyme (0.5 μM ferric or ferrous form) was incubated for 12 hours at 20°C in 0.1 M borate buffer, pH 10.0 in the presence of the indicated concentration of urea. Sample (10 μl) were removed for assay, with the same concentration of urea present in the assay mixture as in the original incubation. The final concentrations of the enzyme and linoleic acid in the assay system were 1.67 nM and 50μM, respectively. In each case the value corresponding to the enzyme in the absence of urea was 1.0. Inset shows the change in emission maximum as a function of urea concentration: (O) the ferrous form, (□) the ferric form of lipoxygenase-1.

Structural changes of soybean lipoxygenase-1 on addition of urea were monitored by fluorescence excited at 280 nm, which detects changes in the environment of aromatic side chains, predominantly tryptophan. The fluorescence emission spectra of lipoxygenase-1 are shown in Figure 2. The native enzyme with no denaturant incubation shows a asymmetric emission band with the maximum emission wavelength at about 332nm. With the increase in the concentration of urea, the emission intensity shows a slightly increase with no fluorescence red-shift at low concentration of urea, then shows a decrease accompanied by a gradual shift in the emission maximum. The typical emission spectra for ferrous form of lioxygenase-1 at 0, 2.0, 4.0, 6.0, 8.0 M urea are as that in figure 2.

The changes in fluorescence of the enzyme at 332nm as a function of urea concentration are also recorded in Figure 1. Both the decrease in emission intensity and the red shift of the emission maximum occur in two stages. With the increase of urea concentration, the emission intensity of the enzyme increases slightly up to 1 M urea, indicating that the conformation of the enzyme molecule as a whole has no apparent changes. The decrease of the fluorescence intensity of the enzyme takes place at urea concentrations higher than 1.0 M up to 2.0 M. With the decrease in the fluorescence emission intensity, there was a gradual red-shift of the emission maximum (to 348 nm at 8.0 M urea), indicating the marked structural changes occurring (inset of Figure 1). Then the spectrum remains almost unchanged when the urea concentration is further increased. Further decrease in emission intensity occur at urea concentration higher than 4.0 M. The shift in the emission maximum is in the similar style as the fluorescence intensity. This suggests that a steady intermediate exists as reported previously [24].

Similar results have been obtained with the ferric enzyme except that the initial drop in the inactivation take place at lower urea concentrations than in the case

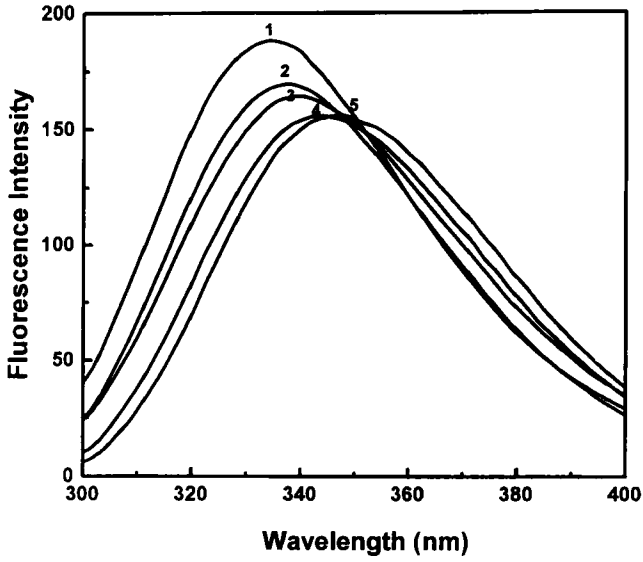


Figure 2. Fluorescence emission spectra of ferrous form of Lipoxygenase-1 denatured in urea solutions of different concentrations. The enzyme concentration is 0.25μM. The concentrations of urea solutions for curve 1-5 are 0, 2.0, 4.0, 6.0, 8.0 M respectively.

of the ferrous enzyme, suggesting that the ferric form of lipoxygenase-1 is more sensitive to the influence of denaturant than the ferrous form.

Kinetics of inactivation and unfolding of soybean lipoxygenase-1

Inactivation kinetics of the ferrous and ferric forms of lipoxygenase-1 were studied by the conventional method. The enzymes were incubated with 6.0 M urea for a definite time interval, and activity assay was started by adding a small volume of the concentrated substrates, linoleic acid, into the incubation system. It can be seen from Figure 3 that the inactivation processes of both the ferrous and ferric forms of lipoxygenase-1 in 6.0 M urea follow single-exponential kinetics. By fitting the

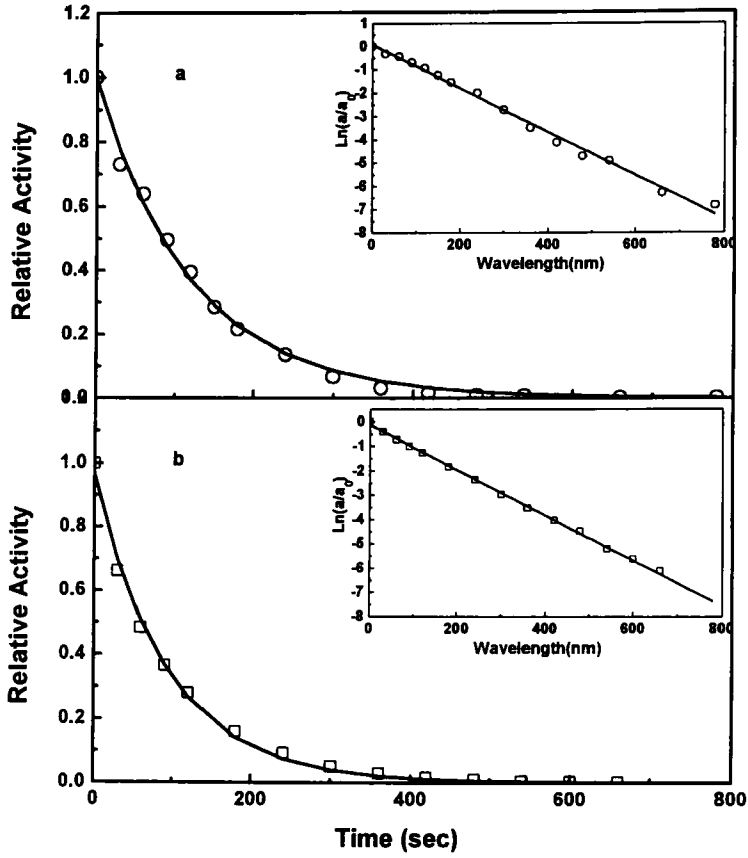
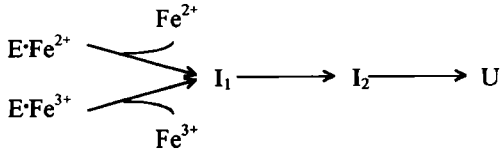


Figure 3. (a) Time-dependence of inactivation of the ferrous lipoxygenase-1 in 6.0 M urea at pH 10.0, 20°C. The solid line represent theoretical curves for a single exponential process. The first order rate constants for generating curves was 0.00934 sec^{-1}. (b) Time-dependence of inactivation of the ferric lipoxygenase-1 in 6.0 M urea at pH 10.0, 20°C. The solid line represent theoretical curves for a single exponential process. The first order rate constants for generating curves was 0.00930 sec^{-1}. The enzyme (5.2 nM ferrous or ferric form) was incubated in the presence of 6.0 M urea. The assay was started at the indicated time by adding a small volume of substrate. The final concentration of linoleic acid was 50μM. The insets show the same data in the corresponding semilogarithmic plots.

experimental data to the equation $v_t = v_0 \exp(-k_{+0}t)$, the first-order rate constants for the ferrous and ferric enzyme were determined to be 0.00934 ± 0.00021 and 0.00930 ± 0.00009 sec^{-1}, respectively.

The unfolding of the ferrous lipoxygenase-1 in 6.0 M urea, as measured by the decrease of fluorescence intensity at 332 nm, is a multiphasic process (Figure 4). By fitting the experimental to equation (1), the three rate constants of enzyme unfolding were determined to be k_1=0.0066 ± 0.0014, k_2=0.0013 ± 0.0004, k_3=0.0003± 0.0002 sec^{-1}, respectively. Compared with the inactivation rate constant of the ferrous enzyme, the fast phase rate constant of unfolding is almost the same as inactivation rate constant, while the rate constants of the two slow phase are much less than that of inactivation process. The unfolding of the ferric lipoxygenase-1 in 6.0 M urea displayed a more complex pattern that required a minimum of three exponential terms to obtain an adequate fit. A typical trace of the unfolding process is also shown in Figure 4. The fluorescence intensity first increase rapidly with a rate constant, k_1, of 0.0101± 0.0009 sec^{-1}. This burst in fluorescence is then followed by successive decreases in intensity which are well described by two kinetic phases with k_2 = 0.0034± 0.0004 and k_3=0.00023 ± 0.00005 sec^{-1}. It can be seen from the inset of figure 4 that in the presence 6.0 M urea, there was about a 30% decrease in protein fluorescence when the native Fe^{2+} enzyme was converted into the folded Fe^{3+} form. Therefore, the results shown in Figure 4 suggest that the fluorescence property of the first unfolding intermediate falls between the Fe^{2+} and Fe^{3+} forms of the enzyme.

Based on the above observations, a minimal scheme for the unfolding of lipoxygenase-1 induced by urea can be represented as follows



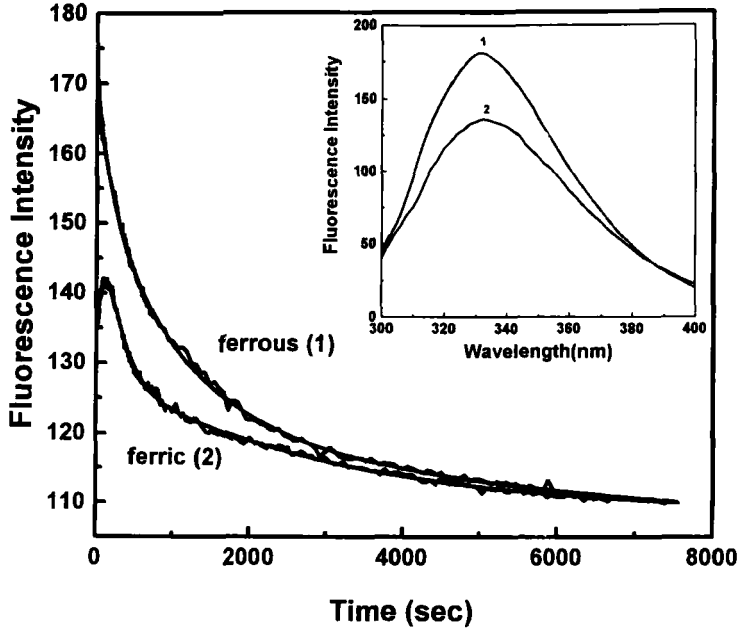


Figure 4. Unfolding kinetics of the ferrous and ferric lipoxygenase-1, as followed by change in fluorescence intensity (excitation=280 nm), in the presence of 6.0 M urea at pH 10.0, 20°C. The enzyme concentration was 0.214 μM. Curve 1 is best fitting result according to eqn.(1) with k_1=0.0066 ± 0.0014, k_2=0.0013 ± 0.004, k_3=0.0003± 0.0002 sec^{-1}, F_1= 13.89 ± 3.19, F_2= 32.32 ± 10.19, F_3= 19.00 ± 3.73, F_∞= 105.40 ± 10.07. Curve 2 is best fitting result according to eqn.(1) with k_1=0.0101±0.0009 sec^{-1}, k_2= 0.0034± 0.0004, k_3=0.00023 ± 0.00005 sec^{-1}, F_1= −57.44 ± 18.34, F_2= 60.10 ± 18.30, F_3= 20.72 ± 2.31, F_∞= 108.20 ± 2.69. Inset: Comparison of the emission spectra of the ferrous (1) and ferric lipoxygenase-1 (2) in the presence of 6 M urea. The fluorescence spectra were scanned immediately after mixing the enzyme with urea solution. The excitation wavelength was set at 280 nm.

where $E{\cdot}Fe^{2+}$ and $E{\cdot}Fe^{3+}$ are the ferrous and ferric forms of lipoxygenase-1, I_1 is the partly unfolded intermediate whose intrinsic fluorescence property lies between the ferrous and ferric forms of lipoxygenase-1, and I_2 and U are the second unfolding intermediate and the final unfolded state, respectively.

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