'Molten-globule state': a compact form of globular proteins with mobile side-chains

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1. INTRODUCTION

The polypeptide chain which composes a globular protein molecule varies its tertiary structure under different solvent conditions. Among these structural states, the native state and the fully denaturated state have been well investigated. In a native state the polypeptide chain folds to a uniquely defined rigid and compact structure, while in a fully denatured state it unfolds into a flexible, swollen, and randomly coiled chain which has very little intromulecular contact and a high degree of internal freedom of motion [1,2].

We would here like to present supporting evidence for the existence of a new type of structural state of the globular protein, which has first been proposed in [3]. In this state the polypeptide chain is supposed to be compactly packed just as in the native state, while intramolecular motions of atoms are extensively released. The state may be called the 'molten-globule state' (the name was contrived during the discussion of Drs O.B. Ptitsyn and C. Crane-Robinson at the International Symposium on Peptides, Polypeptides and Proteins at Galzignano, Padova, Italy, June 20–26, 1982). We describe experimental evidence which proves the existence of this state in cytochrome *c* under the acid perturbation, present a diagram, and discuss its physical and biochemical significance.

2. MATERIALS AND METHODS

Horse cytochrome *c* (Type VI, Sigma) was used throughout this work. For viscosity measurement, cytochrome *c* was dissolved in an aqueous solution without purification. For absorbance and NMR measurements cytochrome *c* was oxidized with ferricyanide. Ferricyanide was then removed using an ion-exchange column (Shatman CM 52) or Amicon membrane filter (UM 10).

The viscosity of the protein solution and the solvent (η_{soln} and η_{solv}) were measured with a rotary viscometer [4]. The reduced viscosity was calculated as $\eta_{red} = (\eta_{soln}-\eta_{solv})/c$, where the typical protein concentration used (c) was 2.0 g/100 ml.

H^1-NMR spectra were measured with a Bruker 360 MHz FT NMR spectrometer. Oxidized and lyophilized ferricytochrome *c* was dissolved into 99.7% D_2O. Indicated values of pD are direct readings of the pH meter.

The diffusion coefficient, D, of the protein molecule was measured with the quasielastic light scattering method [5]. The correlation function of scattered light intensity of 0.5% cytochrome *c* solution was of single exponential form, which shows the monodisperse nature of the protein solu-

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tion. The hydrodynamically equivalent radius of the protein molecule, a, was calculated from the measured value of D, by using the Einstein–Stokes relation,

$$a = kT/(6\pi\eta D) \quad (1)$$

where: k is Boltzman constant; T is temperature; η is the viscosity of the solvent.

Absorbance, CD and fluorescence were measured with a multidimensional spectrometer Mark III, the details of which are described in [6].

3. RESULTS

The horse cytochrome c has been shown to have 3 states in an acidic pH region with moderate ionic strength [7]; the states are designated here as IIa, IIb, and III from low pH. In our experiment, intrinsic viscosity (table 1) shows that the hydrodynamic volume of the protein molecule in state IIb is the same as in state III, while in state IIa the molecule expands greatly. This result agrees with the viscosity measurement in [7]. Furthermore, the equivalent radii of hydrodynamical equivalent spheres obtained from the quasielastic light scattering measurements were 1.98 nm for III and 2.01 nm for IIb (table 1). These results strongly suggest that IIb is as compactly folded as III.

However, as shown in fig.1, the H^1-NMR spectrum of IIb has the characteristic feature of the random coil state [9]. Namely, in IIa and IIb, the

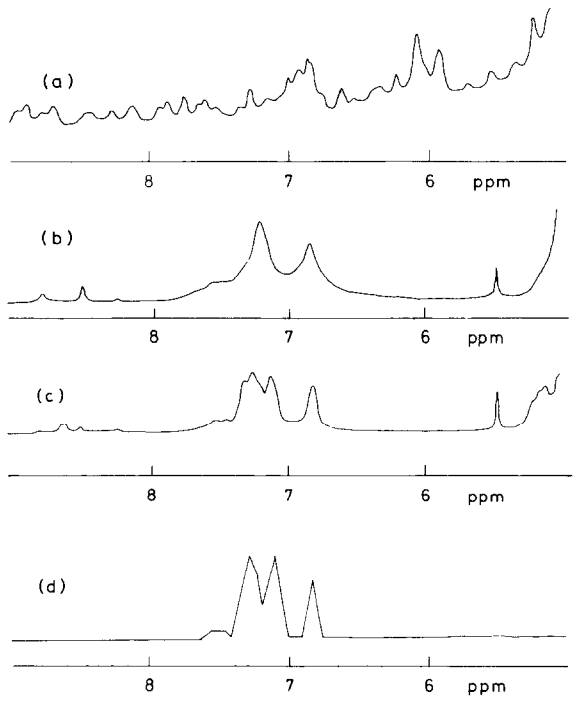

Fig.1. Aromatic region of 360 MHz H^1-NMR spectra of ferri cytochrome c in state III, IIb and IIa. (a) state III (pH 7); (b) state IIb (pH 2.0, 0.5 M KCl); (c) state IIa (pH 2.0, no salt); (d) a superposition of the computed spectra of free amino acids with the same composition of horse cytochrome c. (refigured from [8]). The concentration of protein is 10 mg/ml. Temperature is 293 K.

Table 1

Physical properties of different conformational states of cytochrome c

Conformational state	III	IIb	IIa
Intrinsic viscosity (ml/g)	2.8 ± 0.5[a]	3.1 ± 0.5[a]	12.7 ± 0.5[a]
Equivalent radius (nm)	1.98 ± 0.04[a]	2.01 ± 0.04[a]	–[a]
Relative intensity of fluorescence	2.0 ± 1.0[a,c]	2.0 ± 1.0[a,c]	57.0 ± 1.0[a,c]
Molar ellipticity/residue at 222 nm	10 600[a]	11 300[a]	3800[a]
Heme spin	low[b]	mixed[b]	high[b]
Troughs of CD (280–290 nm)	distinct[a]	weak[a]	none[a]
H^1-NMR (aromatic region)	native-like[a]	random coil-like[a]	random coil-like[a]

[a] See section 2

[b] See [10]

[c] Weak fluorescence indicates that heme and aromatic side chains are close enough for energy transfer [11]. Excitation and emission wavelength are 280 nm and 350 nm, respectively

spectra are close to a superposition of the spectra of free amino acids with the same composition of horse cytochrome *c* (fig.1d). The evidence clearly indicates that the rigidity in intramolecular structure is lost in these states and that internal motion is liberated.

Table 1 provides a comparable list of physical features of III, IIb and IIa. It is evident that IIb is close to III as far as main chain folding is concerned, while the surrounding of the aromatic side chains is isotropic as IIa.

Fig.2 is a phase equilibrium diagram among IIa, IIb, and III states in a pH-ionic concentration plane in an acidic pH. As has already been reported [7], the pH range of state IIb becomes wider as KCl concentration increases. Typical solvent conditions in which one of the phases is dominant are: pH 7, 0.1 M phosphate buffer for III, pH 2.0, 0–1 mM KCl for IIa, and pH 2.0, 0.5 M KCl for IIb. Temperature was 293 K for all the measurements and pH was lowered with HCl.

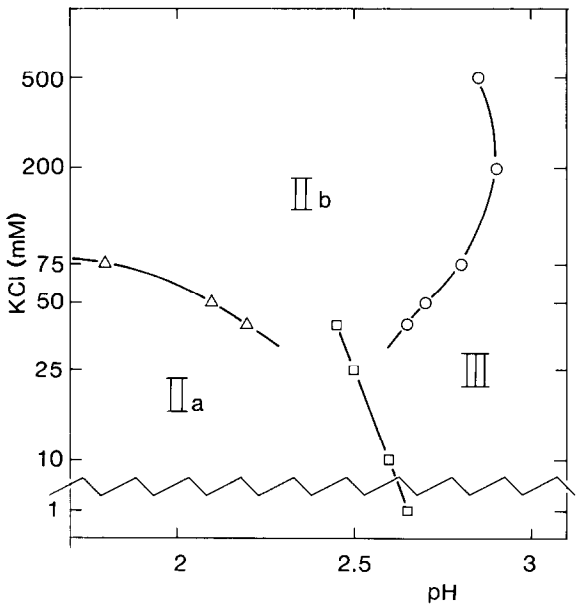

Fig.2. A phase diagram of 3 conformational states of horse ferricytochrome *c* in acidic pH. (III) native state; (IIb) acid denatured state with high KCl concentration; (IIa) acid denaturated state with low KCl concentration. The lines indicate the boundary of phases where the concentrations of two states are equal; i.e., square: IIa = III; triangle: IIb = IIa; circle: IIb = III. Fractions of III, IIb and IIa are determined by absorbance titration. Absorbance at 400 nm and 407 nm, isosbestic points of III and IIb, IIb and IIa, respectively, were used to calculate the fractions.

4. DISCUSSION

In the research on protein conformation change, the main interest has been focused on the unfolding or the refolding of the main chain, so that intermediate states were regarded as a partially unfolded state of the main chain. However, authors in [3] presented another type of ordered structure which situates in between the native and denatured form but without changing its compactness. We confirmed the existence of similar conformational state of the protein molecule, based on the multifaceted observations about state IIb of cytochrome *c*. The molecular characteristics of this state, inferred from intrinsic viscosity and the radius of the protein molecule, and from H^1-NMR spectra are as follows:

(i) the main chain folds as compactly as in the native state;

(ii) intramolecular fluctuation of atoms is distinctly large compared with that in the native state.

The results seem to coincide with a model of 'compact state with fluctuating tertiary structure' which has been proposed in [3] based on the molecular properties of α-lactalbumin in its acid denatured state. Similar findings in different proteins, i.e., the acid denatured state of cytochrome *c* and α-lactalbumin, may indicate that the 'molten-globule state' is a general trend which occurs in globular protein molecules.

Detection of intermediate states in a denatured protein of course totally depends upon the sensitivity and precision of the measurement, and also upon the type of structure-probing observables. The protein has been well known to have a very complex structure, so that it may not be surprising if the existence of internal-phases are disclosed by modern techniques of measurement and data analysis [11–13]. It must be noted here, however, that a strict distinction should be made between the internal phase transition and the change of an observed quantity of a probe that is produced by a trivial effect of the molecular environment, such as solvent perturbation. The former occurs highly cooperatively but this is usually not the case in the latter.

The molten-globule state poses intriguing problems in both physical and biological aspects, especially in the former case. Since it is quite possible that this state is realizable in some physiological conditions, such a liquid-like phase will provide a suitable transit for fast folding of a polypeptide chain into a unique structure [3]. Also, such a state with side chains having high internal freedom of motion might play an important role in efficient enzyme action or in other biochemical functions in globular proteins.

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