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Viscosity-dependent structural fluctuation of the M80-containing Ω-loop of horse ferrocytochrome *c*

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ABSTRACT

To determine the effect of solvent viscosity on low-frequency local motions that control the slow changes in structural dynamics of proteins, we have studied the effects of solvent viscosity on the structural fluctuation of presumably the M80-containing Ω-loop by measuring the rate of thermally-driven CO-dissociation from a natively-folded carbonmonoxycytochrome *c* (NCO-state) in the 0.65–92.5 cP range of viscosity at pH 7.0. At low viscosities (⩽8 cP), the rate coefficient, k_{diss} for dissociation of CO from the NCO-state varies inversely with the viscosity, but saturates at high viscosities, suggesting that CO-dissociation reaction involves sequential stages that depend differently on solvent friction, i.e., solvent coupled and nonsolvent-coupled stages of the process. In the low viscosity regime ($0.65 \leqslant \eta_s \leqslant 8.0$ cP), the rate-viscosity data were fitted to modified Kramers model, $k_{diss} = [A'/(\sigma + \eta_s)^n]\exp(-\Delta G/RT)$, which produced internal friction, $\sigma = 1.35$ cP (±0.88), which suggests that the speed of CO-dissociation from NCO at $\eta_s \leqslant 8.0$ cP is controlled by internal friction.

1. Introduction

Proteins are dynamic molecules which undergo structural fluctuations ranging from ultrafast (in the femtoseconds to picoseconds range) to relatively slow (in the range of seconds) [1–5]. Structural fluctuations that occur on the ultrafast timescales enable a protein to sample a complex conformational energy landscape. These rapid motions ultimately facilitate slower, larger scale protein rearrangements that are accountable for modulating the biological functions [6–8]. The stability and dynamics of a protein in solution are strongly coupled to the dynamics of the solvent [8–12]. The comprehensive investigations of protein dynamics as a function of solvent viscosity have provided insights into fundamental biochemical reactions [12–14] and protein–solvent interactions [5,8–10,15–17].

A common approach for investigating the significance of protein dynamics in a chemical reaction is to study the effect of the solvent viscosity on reaction rate. Kramers theory models the kinetics of chemical reactions and asserts that the reaction rate, k for a heavily damped process depends on both the height of the potential barrier, ΔG and a reaction friction parameter, γ through $k \propto \gamma^{-1}\exp(-\Delta G/RT)$ [18,19]. According to Stokes law, one should expect $\gamma \propto \eta$, which finally gives $k \propto \eta^{-1}$. But this simple behavior is rarely found with protein reactions. Ansari et al. [13] suggested that the total damping rate is a linear combination of the internal (γ_i) and the solvent (γ_s) contributions, where γ_i is the friction coefficient for motion in the protein, and γ_s is the friction coefficient for motion in the solvent, which according to Stokes' law is proportional to its viscosity.

Useful extensions of the Kramers theory to intramolecular activated processes in proteins have been provided by Gavish [20,21], Doster [22], and Schlitter [23]. Gavish [20] has suggested that viscosity plays a vital role in determining the dynamic state of the protein through structural fluctuations. The viscosity-dependent exchange of energy between the protein and the solvent was proposed to be a major source of energy for the protein structural fluctuations in determining its function [20,24]. Since protein structural fluctuations are mainly driven by Brownian motion, therefore, the Kramers theory has been applied to describe the rate of entry and escape of small ligands [12,25] and enzymatic reactions [20,26]. Indeed, several investigators have considered the phenomenological Kramers model to examine the effect of solvent viscosity on several types of protein reactions, including protein folding reactions [27–43], enzyme kinetics [44,45], and the binding of ligands to heme proteins [12,13,21,26,39,46,47]. In particular, for enzyme reactions or protein–ligand interactions, where diffusive motions of protein segments through the solvent alone do not contribute to the observed rates, the rate constant has been found to be inversely proportional to the fractional power of the viscosity, $k \propto \eta_s^{-n}$ $(0 < n < 1)$ [48,49]. The validity of Kramers theory has been tested experimentally for polymer dynamics [50] and is well supported by computer simulations [51,52].

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A large body of work on protein dynamics in viscous solvent indicates that there are three possible origins for fractional viscosity dependence – (i) breakdown of Kramers theory, (ii) the deviation from Stokes law [12,22,34,47,49], and (iii) the reaction involves sequential stages that depends differently on solvent friction, i.e., solvent coupled and nonsolvent-coupled stages of the process [22]. The meaning of fractional exponent, n suggests that the friction coefficient, γ does not vary linearly with the solvent viscosity, η_s. This is essentially a violation of Stokes law ($\gamma \propto \eta_s$) and is not clear at present.

Although commendable progress has been made in the understanding of the effect of solvent viscosity on the fast protein dynamics [53–56], very little has been done to record the effect of solvent viscosity on low-frequency local motion that control the relatively slow changes in structural dynamics of proteins. In this work, we have examined the influence of solvent viscosity on the structural fluctuation of presumably the M80-containing Ω-loop by measuring the rate of thermally-driven CO-dissociation from the natively folded carbonmonoxycytochrome *c* (NCO-state). Because of its smaller size and the content of the heme iron, carbonmonoxycytochrome *c* is a model in experimental protein dynamics studies [41,57–63]. The CO-dissociation rate of NCO-state varies inversely with viscosity initially when the solvent viscosity is low ($\leqslant$8.0 cP), but saturates at higher viscosity, indicating that the CO-dissociation from the NCO-state involves sequential stages that depend differently on solvent friction, i.e., solvent coupled and nonsolvent-coupled stages of the process [22,47]. In the low viscosity regime ($0.65 \leqslant \eta_s \leqslant 8.0$ cP), the rate-viscosity data were fitted to a modified Kramers model, $k_{diss} = [A'/(\sigma + \eta_s)^n]\exp(-\Delta G/RT)$, which produced the internal friction, $\sigma = 1.35$ (±0.88) cP. This suggests that in the low viscosity regime, the speed of CO-dissociation from a native-like folded intermediate, NCO is controlled by internal friction.

2. Materials and methods

Horse cytochrome *c* (Type VI) was purchased from Sigma. Unless otherwise indicated, all the experiments were performed under anaerobic conditions in 15 mM phosphate buffer (pH 7, 25 °C), containing ~2 mM freshly prepared sodium dithionite. The guanidine hydrochloride (GdnHCl) concentrations were determined by refractive index measurements. The compositions of glycerol, sucrose, and glucose are expressed in (w/w)%. We directly measured the kinematic viscosity of glycerol, sucrose, and glucose solutions at 25 °C using a Brookfield digital viscometer (Brookfield Engineering Labs., Inc., USA).

2.1. Measurement of refolding kinetics of unfolded carbonmonoxycytochrome c

Cyt *c* (~0.4 mM), initially unfolded in ~6.5 M GdnHCl, pH 7, was reduced under N_2 by the addition of a concentrated solution of sodium dithionite to a final concentration of 3.0 mM. The unfolded ferrocyt *c* was liganded with CO, and was mixed with the refolding buffer in the stopped-flow in a desired ratio to record refolding kinetics. In each case, before mixing, an equilibration time of ~5 min was given. Stopped-flow experiments used a Biologic SFM400 module. A constant temperature of ~25 °C was regulated by the use of an external water bath. The spectrometer was configured for fluorescence detection (ex: 280 nm; em: 340 nm). A two-syringe mixing (1:7; protein: buffer) at a total flow rate of 8 ml/s was employed. The instrument dead-time for a high-density mixer and a 0.8 mm flow cell was determined to be 3.0 (±0.1) ms. Typically, 10–15 shots were averaged.

2.2. Preparation and identity of native carbonmonoxycytochrome c (i.e., NCO-state)

The NCO-state is prepared from chemically denatured cyt *c* by lowering the denaturant concentration in the presence of carbon monoxide, which binds tightly to the heme iron and prevents formation of the native M80-heme (sulfur–iron) link [57,58,64]. Briefly, cyt *c* is unfolded in the presence of ~6 M GdnHCl. This unfolded protein was deaerated and reduced by adding sodium dithionite. The heme iron of the unfolded protein (called U-state) was liganded with a CO molecule by adding the dry gas at 1 atm pressure to the unfolded protein solution. Unfolded CO-liganded protein (UCO) thus obtained is diluted out with a CO-free refolding buffer (15 mM phosphate, pH 7, 25 °C). The protein chain refolds trapping the CO molecule which is still bonded to the heme iron. Cyt *c* with the trapped CO (called NCO-state) is structurally native-like [59,60] and it represents a long-lived late kinetic intermediate [61,65,66,67]. Folding is blocked as long as the NCO-state stays frozen in the kinetic trap. The folding of NCO to N-state requires the escape of CO molecule from the NCO due to thermal dissociation of the Fe^{2+}–CO bond and simultaneously reorganization of the misconfigured interactions. Actually, the reorganization of the misconfigured interactions involves the ligation of the side-chain of the amino acid residue M80, which is the intrinsic N-state ligand. The N and NCO conformations are not different from each other to any considerable extent [68], and the motional dynamics of the two protein states are not expected to be different either.

2.3. Thermal dissociation of CO from the natively folded carbonmonoxycytochrome c (NCO-state)

Within the limit of the stopped-flow resolution (~3 ms dead-time), the UCO refolds extremely fast in refolding buffer [64]. The kinetics exemplified in Fig. 1c give an observed rate of ~310 s^{-1} in the presence of ~0.3 M GdnHCl. A minor refolding phase of ~40 s^{-1} accounts for remaining 8% of the total observed amplitude arises most likely from a fraction of oxidized protein that does not bind CO. The refolding process UCO → NCO proceed after ~101 fold dilution of the UCO solution with the CO-free folding buffer. Since the concentration of CO in the refolding medium is substantially reduced, and because the affinity of native protein for CO is lower relative to that for the intrinsic M80 ligand, the UCO → NCO process leads immediately to the NCO → N + CO conversion. The NCO → N + CO conversion is basically a unimolecular thermally activated reaction, where the NCO molecules accumulate energy necessary for the CO-dissociation by thermal fluctuation. Collisions between different groups of atoms or structural elements afforded by internal dynamics of the protein act as an intramolecular source of energy. Thermal dissociation of CO from the NCO-state is essentially the Fe^{2+}–CO + M80 → Fe^{2+}–M80 + CO replacement. The Fe^{2+}–CO → Fe^{2+}–M80 conversion does not involve any major conformational adjustment and occurs because of its instability due to greater affinity of M80 sulfur for Fe^{2+} relative to that of CO. Fig. 1d typifies the kinetics of the NCO → N + CO dissociation reaction, recorded after 101 fold dilution of the unfolded CO-liganded protein into a CO-free refolding buffer. The slow increase in absorbance at the heme $\pi \rightarrow \pi^*$ α-band (550 nm) in a single exponential is due to the dissociation of CO (Fig. 1). The slowness of the reaction allows accurate determination of k_{diss} (τ = 23 min with no addition, Fig. 1d) by conventional UV–visible spectrophotometer.

2.4. Equilibrium unfolding measurements of ferricytochrome c (i.e., ferricyt c) in the presence of different composition of glycerol

The GdnHCl-induced unfolding transition curves of ferricyt *c* in the presence of different composition of glycerol solutions were