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ARTICLE

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Light induced states in MbCO denatured with Guanidine hydrochloride

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Abstract We present the results of a comparative study of the binding of carbon monoxide to myoglobin in glycerol/buffer solution with different concentrations of guanidine hydrochloride, under extended illumination over the temperature range 30 – 80 K. The changes in the Soret band indicate that the folding state of the protein is a key parameter in determining the photodissociation process and the relaxation rate of the protein.

Key words Optical spectroscopy · Protein folding · Protein denaturation

Introduction

Study of the light-induced conformations of a protein in the native form is a standard tool for monitoring structural changes related to dynamic states of the protein (Ansari et al. 1985, 1992). A comparison between the photoproducts of the protein in partially unfolded forms can provide important information on the role of folding in the process of ligand rebinding. We have performed our experiment on the myoglobin (Mb), the so called "hydrogen atom of biophysical science".

Myoglobin (Mb) is a small protein that stores and transports dioxygen in muscles. The folded polypeptide chain of Mb embeds a heme group with a central iron atom, bonded with small ligands. In carboxymyoglobin (MbCO) a CO molecule is bound to the heme iron and the covalent bond between the iron and the CO is broken upon absorption of visible light (e. g. a laser flash or extended illumination). MbCO and Mb possess different structures and ligand dissociation initiates a conformational relaxation from a structure still close to the bound state toward the deoxy-Mb structure. Below about 160 K, the rebinding after dissociation is nonexponential in time. Austin et al. (1975) explained this behaviour as due to a static distribution of protein conformations. This implies that the protein ensemble is inhomogeneous with each molecule possessing a different tertiary structure and ligand binding coefficient.

The conformation of the photoproduct is unstable and the protein relaxes to the conformation of the unliganded molecule by a small but global displacement of protein residues near the heme. The changes in the local structure at the iron site were reported first by visible (Iizuka et al. 1974) and X-ray absorption techniques such as EXAFS (Chance et al. 1983; Powers et al. 1984, 1987; Teng et al. 1987) and dispersive XANES spectroscopy (Della Longa et al. 1994), then by crystal diffraction (Schlichting et al. 1994; Teng et al. 1994) and Resonant Raman Spectroscopy (Sage et al. 1995). The protein relaxation after photolysis is reported to be related to changes in the stereochemistry of the heme iron; it is found that the iron moves out of the plane toward the proximal ligand followed by the heme pyrrole nitrogens. This domed equilibrium conformation requires local rearrangement of protein side chains that are packed against the heme. An additional structural change involves the tilt of the proximal histidine ligand away from the heme normal, coupled to a translation of the F helix across the heme as the global protein structure relaxes in response to the conformational change of the heme.

The optical properties of the heme have been extensively studied (Srajer and Champion 1991, Steinbach et al. 1991; Tian et al. 1992, Eaton et al. 1981, Makinen et al. 1983) to investigate the dynamics of the myoglobin. Most of the studies on ligand binding have been carried out in the presence of an organic cosolvent that allows optical monitoring of kinetics over large range of time and temperature. However, it is important to realize that the solvent can perturb both protein structure and its dynamics. The presence of glycerol at low temperature can induce important differences with respect to the aqueous solu-

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tions. The differences have been partially attributed to the suppression of dynamic fluctuations of the protein structure due to high viscosity of the glycerol/water (Ansari et al. 1992; Sage et al. 1995). Moreover, the mechanism of the kinetics and the binding rate will depend on the *folding state* of the protein. Therefore, modifications of the heme pocket size and the interaction between the heme and the polypeptide chain induced by the presence of a denaturing solvent, such as guanidine-hydrochloride (Gdn-HCl), can alter the photodissociation process.

In this work the photolysis of native MbCO as well as denatured MbCO (with Gdn-HCl) has been performed to study the influence of the protein folding state on the ligand recombination. We have observed effects of the folding conditions on protein structure and dynamics of MbCO, in the temperature range 30 K < T < 80 K, by means of time and temperature dependent analysis of the Soret band.

Materials and methods

Lyophilized horse-heart myoglobin was purchased from Sigma Chemical Co. (St. Louis MO U.S.A.) and was used without further purification. Glycerol (Fluka, Buchs, Switzerland) and all other chemicals were of analytical grade. Stock solutions were prepared by dissolving an appropriate amount of lyophilized Mb in 0.2 M phosphate buffer (Na_2HPO_4 + NaH_2PO_4 H_2O) pH 7 (in water at room temperature), 65% v/v glycerol, with a final protein concen-

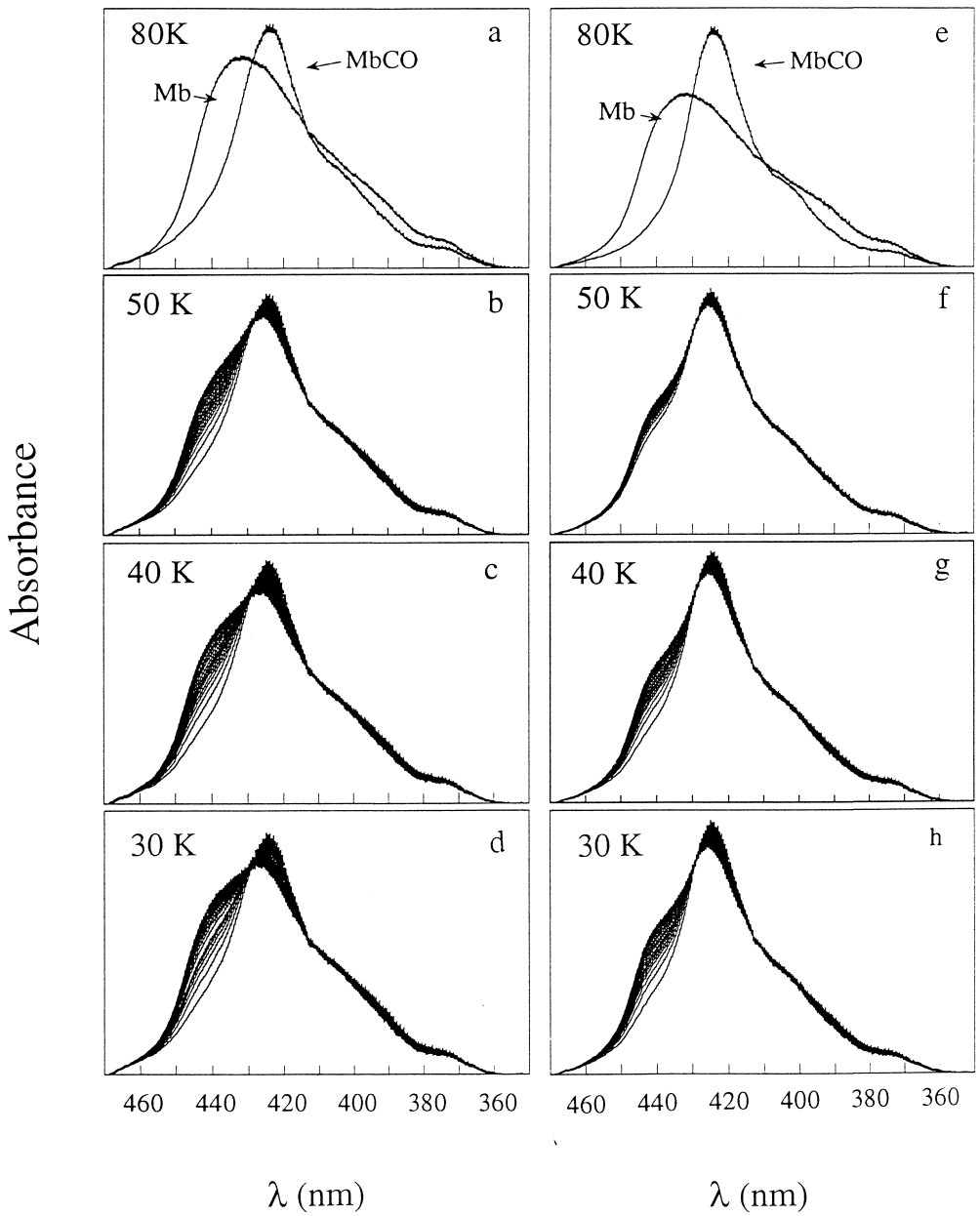


Fig. 1 a–h Experimental plot in the Soret band region (350–470 nm) obtained using the OMA software. In the upper part of figure the unphotolyzed MbCO and Mb spectra at T = 80 K in the native form (panel **a**) and denatured with 1.4 M Gdn-HCl (panel **e**) are compared. Optical spectra of native MbCO at T = 30 K (panel **d**), T = 40 K (panel **c**), T = 50 K (panel **b**), and MbCO + 1.4 M Gdn-HCl at T = 30 K (panel **h**), T = 40 K (panel **g**), T = 50 K (panel **f**). Each curve represents the sample's state after 3,97 s of continuous illumination

tration of $1.2 \cdot 10^{-5}$ M. MbCO samples were subsequently obtained by adding a slight excess of dithionite under anaerobic conditions and saturating with CO. The exact concentration was determined with a diode array spectrophotometer (model 8452A H. P.), using a quartz cell (1 mm by 10 mm pathlength). The partially denatured state was obtained by adding the required concentration of Gdn-HCl (0.5 M, 0.8 M, 1.4 M, 1.8 M).

The sample was transferred into a brass cell (5 mm pathlength), immediately after preparation. The sample holder was mounted in a closed-cycle helium refrigerator (Leybold, model ROK 10-300) where the cryostat was equipped with mylar windows and a temperature controller (Leybold, Variotemp model HR1). Spectra in the 350–470 nm range were measured with an OMA EG & G (model 1232) spectrophotometer, with different acquisition modes: for static data 1 memory averaging 35 scans (following 3.97 s of illumination); for dynamic data (during the photodissociation process) 50 memories collected with the same statistic.

Spectra were acquired at room temperature and every 10 K between 100 K and 30 K. The sample was photolyzed by continuous illumination from a 12 V–100 W QTH Oriel lamp (model n. 6333). The following photolysis protocol was performed: for measurements between 30 K and 70 K the lamp was turned on with concomitant data acquisition. The photolysis process was monitored at 424 nm for the MbCO state and 439 nm for the deoxy-Mb state. In this temperature range, the photolysis process reached steady state after 200 s of illumination. This equilibrium state was confirmed at each temperature by the spectra recorded after 600 s. The sample was heated to 140 K after the acquisition to ensure complete rebinding and relaxation. The baseline was measured using mylar windows and buffer solutions. No baseline variation was observed in the 350–450 nm region in the whole temperature range explored. For all measurements the mylar baselines were recorded at room temperature and substracted from the measured myoglobin spectra before further analysis.

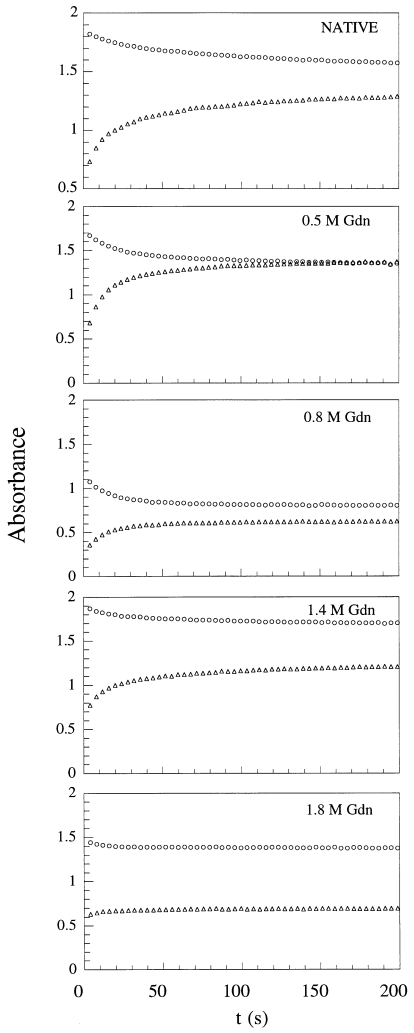


Fig. 2 Optical density of the native and denatured forms of myoglobin as a function of the illumination time at 30 K. Upper curve (circles) shows the variation of the optical density at 424 nm (MbCO peak) as function of the time and the lower curve (triangles) shows the variation of the optical density at 439 nm as function of the time

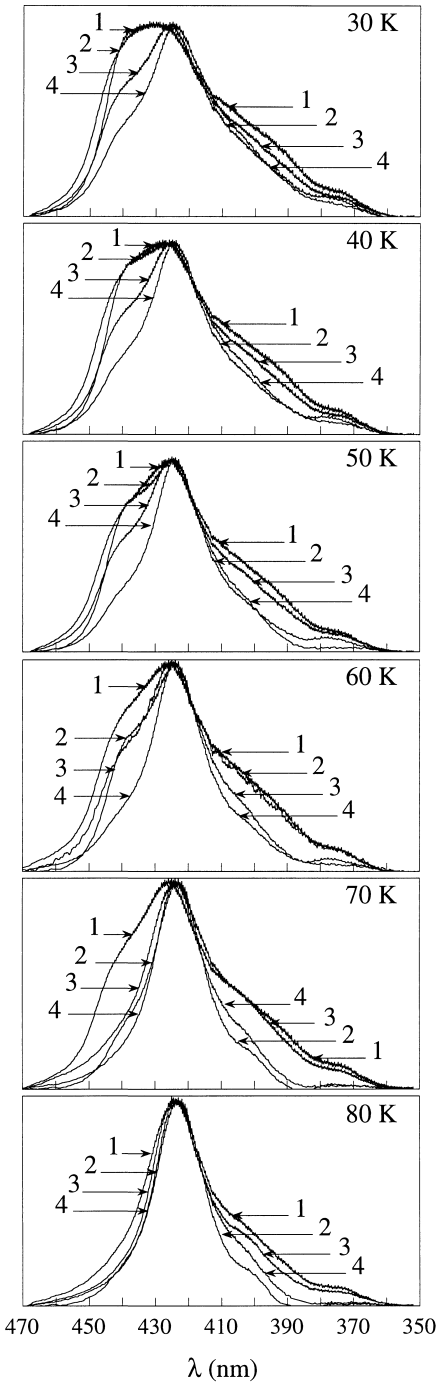
Results

In order to study the kinetic mechanism involved in the photolysis processes of horse-MbCO, optical spectra of the protein under native and denaturing conditions were collected as a function of both Gdn-HCl concentration and temperature.

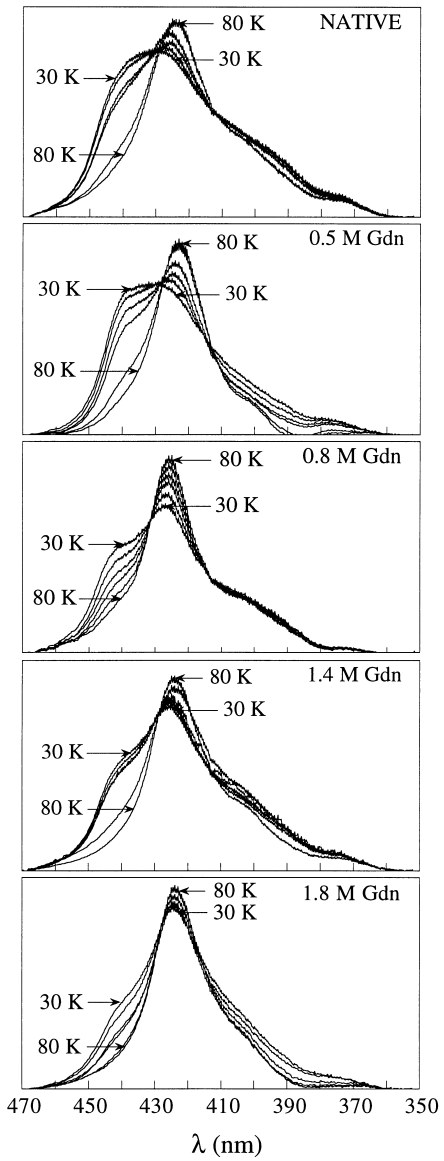
In Fig. 1, the Soret spectra at 30 K, 40 K, 50 K, and 80 K of the native protein and the partially denatured protein (with 1.4 M Gdn-HCl), are compared. The spectra follow the photolysis process up to steady state (200 s), each curve refers to the sample's state after an additional 3.97 s of continuous illumination. As previously reported (Iizuka et al. 1974; Srajer et al. 1986; Tian et al. 1992) the photodissociation process is revealed in the wavelength shift of the peak in the Soret range (350–500 nm) from 424 nm (related to MbCO state) to 432 nm (related to deoxy-Mb form). The wavelength shift of the MbCO peak towards the deoxy state is clearly visible. For comparison, the MbCO and Mb unphotolyzed spectra at 80 K in the native state and denatured with 1.4 M of Gdn-HCl are reported (upper part of the figure).

In Fig. 2 the absorbance of the native protein and of the denatured forms of myoglobin as function of the illumination time at 30 K are reported. In each panel, the upper curve shows the variation of the absorbance at 424 nm (corresponding to the MbCO peak), and the lower curve shows the variation of the absorbance detected at 439 nm, as a function of time. The photolysis rate of 0.5 M Gdn-HCl is higher than at the other concentrations.

Figure 3 shows optical spectra at steady state (after 600 s of photolysis) as a function of the Gdn-HCl concentrations (Fig. 3a) and the temperature (Fig. 3b). At tem-



a



b

Fig. 3 **a** Optical spectra of protein after 600 s of photolysis. Each curve, normalized to unity, is referred to a fixed Gdn-HCl molarity (1=native, 2=0.5 M, 3=1.4 M, 4=1.8 M), each panel to the relative temperature values (80 K – 70 K – 60 K – 50 K – 40 K – 30 K). **b** Optical spectra of protein after 600 s of photolysis. Each curve is referred to a fixed temperature (from top to bottom: 80 K – 70 K – 60 K – 50 K – 40 K – 30 K), each panel to the Gdn-HCl molarity (native, 0.5 M, 0.8 M, 1.4 M, 1.8 M)

peratures higher than 70 K no photodissociation effects are evident because of the fast rebinding rate.

By comparing these spectra with those of deoxy and carboxy myoglobin in the native and denatured form we can estimate the percentage of MbCO. Figure 4 shows the percentage of MbCO present in the sample after 600 s of continuous illumination at different temperatures for a fixed value of the denaturant concentration. The percentage is calculated by considering the measured spectrum as a superposition of the MbCO and Mb deoxy spectra

$$A(\lambda, t) = [\mathrm{MbCO}]\,(t)\; A_{\mathrm{MbCO}}(\lambda) + [\mathrm{Mb}]\,(t)\; A_{\mathrm{Mb}}(\lambda)$$

At a fixed temperature the MbCO percentage is found to increase with the Gdn-HCl concentration. The increase of MbCO, less pronounced at higher Gdn-HCl's molarities, is certainly related to the different folded state and to the different structure of the active site in the native and denatured states of the protein.

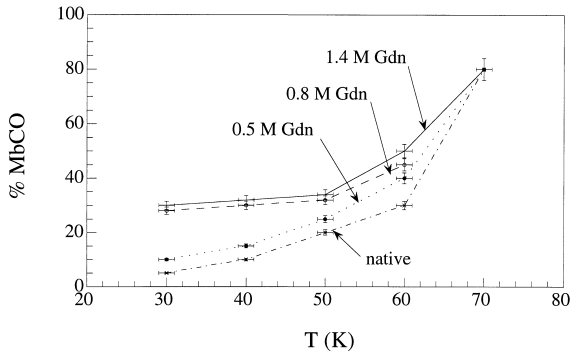


Fig. 4 MbCO percentage still present in the sample (after 600 s of continuous illumination) as function of the temperature at a fixed denaturant concentration: –+– = 1.4 M Gdn-HCl; –○– = 0.8 M Gdn-HCl; –●– = 0.5 M Gdn-HCl; –×– = native

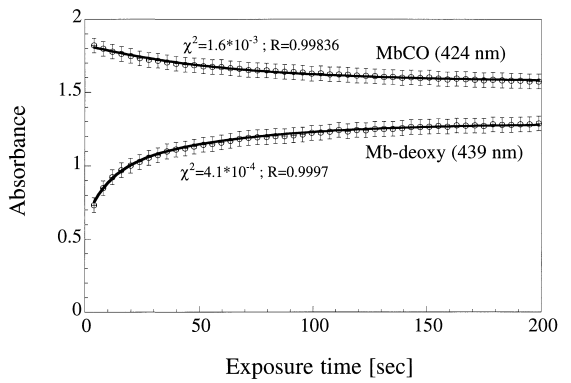


Fig. 5 Theoretical fit of the optical density variation of MbCO (monoexponential behaviour) and Mb-deoxy (double exponential behaviour) curves, as function of the time. The CHI-square values determined from the fitting procedures are also reported. These data are deduced from the spectrum of the photolyzed sample in the native from at T = 30 K. Symbols: experimental data; solid line: theoretical curve

Discussion

During the photolysis process the following relationship is always valid:

$$N_{bound} = N_{total} - N_{unbound}$$

where N_{bound} represents the number of molecules bound to the ligand CO, N_{total} the total number related to the initial population of MbCO molecules, and $N_{unbound}$ the number of dissociated molecules.

The factor dN_{total}/dt (where t is the exposure time) is obviously zero because the total number of molecules must remain constant, while dN_{bound}/dt and $dN_{unbound}/dt$ depend on the degree of denaturation and the temperature, i.e. the process starts with a rapid decrease of the N_{bound} during the first second of illumination and slowly proceeds to a steady state. Consequently the parameter $N_{unbound}$ shows an increase and then becomes saturated.

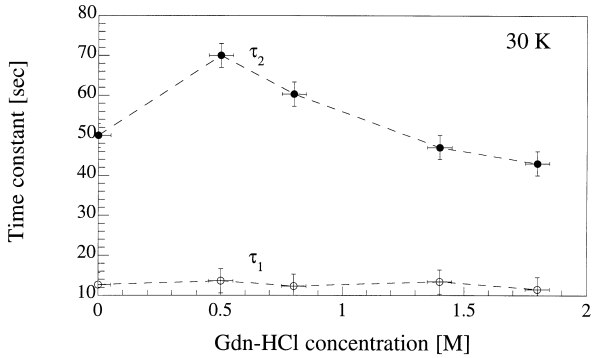


Fig. 6 Variation of the parameters τ_1 and τ_2 as function of the Gdn-HCl concentration. Dashed lines are drawn to guide the eye

Both the unphotolyzed molecules and the molecules that have rebound a ligand contribute to N_{bound} (the number of MbCO molecules) while only the photolyzed molecules contribute to $N_{unbound}$.

The intensity dependence of the peaks of deoxy and MbCO as a function of illumination time is reported in Fig. 2, where each experimental point corresponds to 3.97 s of photolysis. The entire process covers a period of 200 s. The saturation effect is clearly visible in the figure. For T > 70 K no photolysis process can be seen because the rebinding mechanism is too fast.

The curve for the MbCO peak can be fitted by an exponential equation, while a function with two exponential factors is required for the Mb deoxy peak; this can be written, as reported in previous work (Frauenfelder et al. 1991), as:

$$A_{Mb} = [1 + \gamma - \alpha \exp(-t/\tau_1)]/ \\ [1 + \beta \exp(-t/\tau_1) - \beta \exp(-t/\tau_2)] \quad (1)$$

where α and γ are the normalization factors, β is the weight factor for the second exponential and τ_1 and τ_2 are the corresponding time constants. An example of a fit of the optical density variation to Eq. (1) is shown in Fig. 5.

If A_{MbCO} is the population of the bound state, A_{Mb} the population of the photolyzed state, assuming a single first order rate coefficient and that at t = 0 all proteins are in the bound state ($A_{MbCO} = 1$), the kinetic process is described by

$$A_{MbCO}(t) = [k_r + k_l \exp(-(k_l + k_r)\, t)]/(k_l + k_r) \\ A_{Mb} = 1 - A_{MbCO} \quad (2)$$

where k_l is the rate of the photolysis, k_r the rate of the rebinding and $\lambda = k_l + k_r$ is the exponential rate.

The steady state population is given by

$$A_{Mb}(\infty) = 1/[(k_l/k_r) + 1] \, .$$

From Fig. 4, the deduced values of k_l/k_r indicate that in this temperature range (30 K < T < 70 K), the exponential is dominated by k_l (corresponding to τ^{-1} in Eq. (1)).

The single exponential of A_{MbCO} is in accord with this assumption. On the other hand, the A_{Mb} variation, fitted by two exponentials, suggests that it is sensitive to a sec-

ond process which can be attributed to a light-induced relaxation into a long lived state (Srajer et al. 1991; Nienhaus et al. 1994). Therefore, on the basis of recent results (Post et al. 1993; Di Iorio et al. 1991; Doster et al. 1989), we can hypothesize that a fraction of any population of unbonded protein molecules is in an intermediate state that coexists with the molecules in the definitive state. The τ_1 and τ_2 parameters are related respectively to the initial fast process and to the slow process (toward a definitive light-induced state). The occurrence of two processes is related to the fact that some regions of the protein (involving small groups of atoms) might fluctuate rapidly, while the other regions (involving larger groups of atoms) might undergo a relatively slow interconversion (Tian et al. 1992). When the Fe-CO bond is broken, the structural change is communicated to the protein through the proximal histidine. Because the light induced relaxation of the heme is coupled to the rearrangement of adjacent side chains and a relaxation of the polypeptide backbone, the time of the relaxation to the final state varies with the folded state of the protein. Therefore, the τ_2 is related to the Gdn-HCl concentration: in a folded state (or in a compact structure) the τ_2 is higher with respect to the one in a partially unfolded state.

The values of τ_1 and τ_2 extracted from Eq. (1) are reported as a function the Gdn-HCl concentration at T = 30 K in Fig. 6. The τ_1, which depends on the number of absorbed quanta and the quantum efficiency, is independent of the Gdn-HCl concentration, while τ_2 has been found to be strongly dependent.

An important feature can be pointed out: at 0.5 M Gdn-HCl the τ_2 is higher than for the native sample (τ_2 in 0.5 M Gdn-HCl is one and a half times higher). Consequently, the relaxation process to a deoxy-like heme pocket conformation, which involves the polypeptide chain, is longer. This could be attributed to a more compact structure of the protein with respect to the native sample.

Analogously the 0.8 M Gdn-HCl sample shows an intermediate compact structure between the 0.5 M state and the native protein. On the other hand for samples with a concentration higher than 1 M Gdn-HCl, the heme-pocket is open and the relaxation process for the photolyzed molecules is shorter. This is in agreement with the previous reports (Pace 1986; Hagihara et al. 1993), where Gdn-HCl at low concentration is found to act as a source of anion binding to the positively charged groups of the protein. This results in a screening of the repulsive effects responsible for the unfolding and therefore induces a more compact protein structure. At higher concentrations, the classical denaturing effect becomes dominant and unfolding occurs, creating a more open structure for the active site.

In conclusion, our experimental data indicate that the folding state of a protein is a key parameter in determining the structural fluctuations and the relaxation time. A more compact conformation of the protein allows a faster process of photolysis with respect to the partially unfolded one, but increases the relaxation time for the unligated molecules to the light-induced long lived state.

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