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Journal

Biochimica et biophysica acta, 913(2)

ISSN

0006-3002

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Publication Date

1987-06-01

DOI

10.1016/0167-4838(87)90324-4

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Dynamic aspects of the heme-binding site in phylogenetically distant myoglobins

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(Received 15 July 1986)

(Revised manuscript received 17 November 1986)

Key words: Myoglobin; Time resolved fluorescence; Dipolar relaxation; Fluorescence; Heme binding site

Dynamic aspects of the heme-binding site of myoglobins derived from two phylogenetically distant species, namely sperm whale and bluefin tuna, have been investigated by studying steady-state and time-resolved emission properties of 2-*p*-toluidinyl-6-naphthalene sulfonic acid (TNS) apomyoglobin conjugates. Multi-frequency phase and modulation fluorometry data indicate that charge movements occur in the fluorophore environment during the excited state lifetime in the sperm whale myoglobin system. In the case of the bluefin tuna myoglobin TNS adduct these movements were not detected, indicating that the relaxation processes differ in the two types of myoglobins.

Introduction

The dynamic nature of proteins is now well established, although the functional significance of the various dynamic processes is less clear. The possible functional importance of protein dynamics in enzyme catalysis and ligand-binding processes is an area of active interest [1–5]. The wealth of structural and kinetic data on the heme-binding proteins, myoglobin and hemoglobin, and the likely importance of dynamic processes in these proteins have prompted us to investigate dynamic aspects of the heme-binding

site in two distinct apomyoglobin-fluorophore conjugates. The two myoglobins we have chosen, from sperm whale and bluefin tuna, are quite distant from an evolutionary perspective. The heme pocket in these proteins is formed from two adjacent domains [6–8].

Fluorescent probes such as ANS, TNS and DANCA have been shown to bind to the heme-binding site of apomyoglobins [9–12]. The utility of these naphthalene-based probes in such studies derives from their large excited-state dipole moments [13]. The sudden change in the charge distribution in the fluorophore upon excitation can induce rearrangements of charged groups and dipoles in the immediate vicinity of the probe – the rearrangements occur as the system proceeds to a minimal energy state. Such charge and dipole rearrangements produce alterations in various steady-state (spectral) and dynamic (time-resolved) emission properties.

If we consider the absorption properties of such

Abbreviations: ANS, 1-anilino-8-naphthalene sulfonic acid; TNS, 2-*p*-toluidinyl-6-naphthalene sulfonic acid; DANCA, 2-dimethylamino-6-naphthoyl-4'-cyclohexane carboxylic acid.

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fluorophores in polar media we note that the red region of the final absorption band originates from molecules existing in lower energy states, i.e., fluorophores surrounded by solvent molecules which are energetically favorably disposed with respect to the probe's ground state dipole moment. Similarly, the red region of the emission spectrum originates from fluorophores existing in a minimal energy condition, i.e., the case in which the solvent molecules have totally relaxed around the excited-state dipole moment. In these cases, then, the emission spectrum changes as the excitation wavelength is altered. Hence, excitation of such probes in the blue region of the absorption band, which selects molecules with energetically unfavorable ground-state environments, and observation of the fluorescence from the red region of the emission band maximizes the potential for observing solvent relaxation processes. Conjugates of naphthalene probes with proteins can thus provide information on dynamic processes in the protein matrix surrounding the probe. In the present study of TNS-apomyoglobin conjugates we specifically address the question of dynamic processes in the heme binding site.

Steady-state spectral observations alone are not sufficient to demonstrate unequivocally the existence of dynamic processes, since a distribution of binding sites corresponding to different static energy states will also give rise to such excitation wavelength dependency. The time evolution of the spectral properties will, however, convey information about the dynamic processes such as dipolar relaxation. Dipolar relaxation of the protein matrix around the excited probe dipole may arise from motions of the peptide backbone (since the amide linkages have dipole moments) or amino acid side-chain residues, in particular residues with charges or permanent dipoles. Water molecules near the fluorophore may also exhibit relaxation. The technique of multifrequency phase and modulation fluorometry is well suited for investigations of such dynamic processes in proteins [14–16]. In particular this technique permits us to study processes occurring in the picosecond to microsecond time scale. However, the time scale of the excited-state lifetime being utilized, in this case on the order of 10^{-8} s, will determine the time scale of the observable relaxation processes.

Materials and Methods

Tuna myoglobin was prepared as previously described [17]. The homogeneity of the preparations was evaluated by SDS-polyacrylamide gel electrophoresis at pH 8.6 in 10% gels; a single protein band was always observed under these conditions. Sperm whale myoglobin, purchased from Sigma Chemical Co. (St. Louis, MO) was further purified by column chromatography on Sephadex G-50 (2.5×100 cm). The apoproteins were prepared by the butanone extraction technique [18]. The apomyoglobin solutions did not exhibit any sensible absorption in the region of the Soret band, indicating the absence of significant amounts of heme. The final buffer solution used was always 0.05 M sodium phosphate in 0.1 M NaCl, (pH 7.0). The molar extinction coefficients at 280 nm of the apomyoglobins were calculated from the tryptophan and tyrosine content using molar extinction coefficients for these residues of 5500 and 1250, respectively [19] and were found to be 13 500 for sperm whale and 8000 for tuna apomyoglobin. TNS, from Molecular Probes (Junction City, OR) was recrystallized twice according to the procedure of Gafni et al. [11]. The molar extinction coefficient at 350 nm for TNS is 6640 [11]. The TNS/apoprotein molar ratio was maintained at less than 1/50 to minimize any contribution from non-specifically bound fluorophore. Steady-state fluorescence spectra were obtained using a Perkin-Elmer MPF-44A spectrofluorometer. A thermostated cell holder maintained the temperature at 20°C; excitation and emission bandwidths were 5 nm. All solutions were passed through a millipore filter prior to use to eliminate aggregated material; the fluorescent background from comparable unlabeled samples was insignificant. All experiments were carried out using freshly prepared solutions of apomyoglobin.

Results

Lifetime data were obtained using the multifrequency phase and modulation fluorometer described by Gratton and Limkeman with modulation frequencies ranging between 10 and 150 MHz [20]. The fluorometer was equipped with an ISS-ADC (I.S.S., Inc., Champaign, IL) interface

for data acquisition. The samples were excited at 325 nm using a He-Cd laser (Liconix model 4240N, Sunnyvale, CA). Emission was observed through an interference filter with a center wavelength transmittance at 510 ± 2 nm and a full width at half maximum of 10 nm (Corion Co., NJ). In phase fluorometry a lifetime measurement consists of a set of values of the phase shift, P , and the demodulation, M , of the emission with respect to the excited light at several different modulation frequencies. Two independent determinations of the lifetime are obtained from the phase (τ^P) and from the modulation value (τ^M), respectively

$$\tau^P = \tan(P)/2\pi f$$

$$\tau^M = [1/M^2 - 1]^{1/2}/2\pi f$$

where f is the frequency of light modulation. For a single exponential decay τ^P and τ^M have the same value, irrespective of the modulation frequency. For a decay due to a sum of independent exponentials $\tau^P < \tau^M$, whereas for a decaying system in which there is an excited state reaction it is possible to have $\tau^P > \tau^M$. In this latter case the value of the phase can exceed 90° and the apparent phase lifetime can be negative. Phase and modulation data were analyzed using a non-linear least-squares routine described elsewhere [21,22] which fits the decay to a sum of exponentials.

Sperm whale apomyoglobin is known to bind TNS in the same site which accommodates the heme moiety and with the same stoichiometry, i.e., 1:1, with a dissociation constant of $5.2 \cdot 10^{-6}$ M [11]. Tuna apomyoglobin binds TNS in the same 1:1 stoichiometric ratio with a slightly lower affinity ($K_d = 1.5 \cdot 10^{-5}$ M). When the dye is bound to the globin, its quantum yield is about 50-fold higher than in water with a concomitant large blue shift of the emission maximum [23]. Fig. 1 shows the corrected emission spectra of the TNS conjugates of sperm whale and tuna apomyoglobin. The spectrum of the latter displayed a significant red shift of the emission maximum, i.e., 7 nm.

For the time-resolved measurements excitation was at 325 nm while emission was observed at 510 nm; these wavelengths corresponded to the blue region of the absorption and to the red region of the emission, respectively. In the case of sperm

Fig. 1. Emission spectra of TNS in apomyoglobins. Left curve: sperm whale apomyoglobin. Right curve: tuna apomyoglobin. The excitation wavelength was 325 nm.

whale TNS-apomyoglobin the phase lifetime became longer than the modulation lifetime as the frequency was increased beyond 30 MHz (Table I). At very high frequencies, i.e., frequencies higher than 120 MHz, the phase angle exceeded 90° and the apparent phase lifetime became negative. The later observation demonstrates the occurrence of excited-state processes [15,24]. Similar results have

TABLE I

PHASE, MODULATION AND LIFETIME DATA FOR SPERM WHALE TNS-APOMYOGLOBIN AT DIFFERENT MODULATION FREQUENCIES

F (MHz)	Phase (deg)	Modulation	τ^P (ns)	τ^M (ns)
10	32.8	0.830	10.24	10.68
20	51.6	0.614	10.04	10.22
30	62.5	0.472	10.21	9.91
40	69.1	0.370	10.43	9.99
50	78.4	0.258	12.94	9.93
85	84.1	0.185	18.22	9.95
120	89.8	0.134	616.2	9.83
150	92.5	0.107	-24.7	9.83

been reported by Gafni et al. [11] and by Lakowicz et al. [25] for the same protein conjugate. This observation is also confirmed by the non-linear least-square analysis (Table II) which showed the presence of a lifetime component with a negative fractional intensity [26].

The results for the tuna TNS-apomyoglobin conjugate were quite different. The frequency-dependent phase and modulation data for this conjugate are reported in Table III. We note that the phase lifetimes were, in all cases, shorter than the modulation lifetime. Moreover, both phase and modulation lifetimes decreased as the modulation frequency increased. These observations are consistent with the presence of a heterogeneous emitting population [15]. The non-linear least-squares analysis of the data did not give any evidence of lifetime components with a negative fractional intensity (Table II).

The blue shift of the emission band of TNS bound to sperm whale apomyoglobin compared to that observed for the tuna apomyoglobin case may originate as a consequence of either static or dynamic considerations. On average, the heme pocket in tuna myoglobin would appear to be more polar than that of the sperm whale myoglobin, since its corresponding TNS spectrum is red shifted. The lifetime data in the red region of the emission band for the sperm whale TNS-apomyoglobin system suggest, however, that dipolar relaxation processes in the heme binding site occur on a time scale comparable to the fluorescence lifetime. The lack of such relaxations for the tuna apomyoglobin case indicates that either the dynamic processes are absent or that they occur on a time scale either

TABLE III

PHASE, MODULATION AND LIFETIME DATA FOR TUNA TNS-APOMYOGLOBIN AT DIFFERENT MODULATION FREQUENCIES

F (MHz)	Phase (deg)	Modulation	τ^P (ns)	τ^M (ns)
10	31.1	0.828	9.60	10.77
20	48.3	0.619	8.93	10.11
30	56.5	0.471	8.00	9.95
40	60.8	0.376	7.12	9.80
60	66.0	0.275	5.95	9.28
90	68.9	0.194	4.59	8.94
120	69.6	0.148	3.56	8.66
150	70.3	0.123	2.96	8.56

too fast or too slow, relative to the fluorescence lifetime, to be observed.

Discussion

There are several observations in the literature which suggest that the heme-binding site of tuna apomyoglobin may have more flexibility than the corresponding site in sperm whale apomyoglobin. For example, the α -helical content of tuna myoglobin is considerably less than that of sperm whale myoglobin [27]. Moreover, the loss of helical structure upon removal of the heme from tuna myoglobin is on the order of 50% but only 15% for sperm whale myoglobin [27]. Additionally, the rotational strength of the dichroic band acquired by the heme in the native structure of tuna myoglobin is lower than that observed for sperm whale myoglobin. This latter result indicates that the aromatic side chains which interact with the heme moiety in the tuna myoglobin are in a less rigid environment than those responsible for the Soret circular dichroism of sperm whale myoglobin [28].

From the steady-state spectral data alone, i.e., the red-shifted emission of the tuna myoglobin spectrum with respect to sperm whale myoglobin spectrum, one might conclude that dipolar relaxation processes are present in the tuna apomyoglobin conjugate. However, the time-resolved measurements do not give evidence for such relaxation process. The existence of extensive lifetime heterogeneity in this case, however, suggests that a num-

TABLE II

LIFETIME ANALYSIS: THREE EXPONENTIAL COMPONENTS ANALYSIS

Protein	Lifetime (ns)	Fraction
Sperm whale	15.98	0.264
	8.61	0.754
	0.10	-0.018
Tuna	10.79	0.896
	3.18	0.074
	0.37	0.030

ber of distinct conformational substates exist for TNS in the heme-binding site. (We note that TNS in isotropic non-viscous solvents exhibit a single exponential decay.) The observed difference in the relaxation behavior between tuna and sperm whale myoglobin then support the viewpoint that spectroscopic parameters associated with the relaxation process are dominated by local effects as opposed to general dynamic features such as protein backbone motions or movements of bound water molecules. Presumably, these general dynamic features are similar in the two myoglobins. Instead, we suggest that a particular protein residue or a water molecule is properly positioned in sperm whale myoglobin to interact with the TNS excited-state dipole. In tuna myoglobin instead, there are several different protein surroundings or microenvironments for the TNS molecule as shown by the lifetime heterogeneity. Consequently, in some of the TNS apomyoglobin substates in tuna protein, the excited-state dipole orientation, relative to the surrounding protein matrix, might not be properly positioned to be sensitive to dipolar relaxation processes. Alternatively, the specific group responsible for the relaxation process in sperm whale myoglobin may be absent in the tuna protein.

Acknowledgements

This work was supported in part by Grant PCM-8403107 from the National Science Foundation (E.G.), Grant I-986 from the Robert A. Welch Foundation (D.M.J.) and a grant from The Research Corporation (D.M.J.).

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