with large entropic contributions to hydrophobic binding in cyclodextrins.[2] Furthermore it is sufficient that the few enthalpy rich water molecules within the cavity, which lack several stabilizing hydrogen bonds, are replaced by a still highly mobile lipophilic substrate part. Similar reasoning has been applied earlier for explaining the often stronger binding of phenyl derivatives to β-CD in comparison to α-CD.[2a,e]

Experimental and Computational Details

α- and β-Cyclodextrins are commercially available (Fluka); γ-cyclodextrin was a gift of Professor Szejtli, Budapest. The cycloamyloses were dried prior to use at 100 °C over phosphorus pentoxide in vacuum and found to be at least 95% pure, based on ^{1}H-NMR signal comparison with added methanol as internal reference of known concentration. Only commercial γ-cyclodextrin from other sources showed other signals due to contaminations. ANS in the form of its ammonium salt (Sigma) was used as purchased, the TNS potassium salt (Serva) was dried at 100 °C prior to use. Equilibrium titrations were carried out similarly as described earlier[10] by using stock solutions of suitable concentrations in reagent grade solvents.

In a typical NMR or fluorescence titration 0.5 mL of a CD stock solution (e.g., 10^{-3} M) was added in eight steps to a solution of the substrate dye (for fluorescence, 2 mL, 1.00×10^{-4} M; for NMR, 0.5 mL, 10^{-3} M), leading to complexation degrees from ~20% to ~80%.

Fluorescence measurements were carried out with a Hitachi instrument F-3000 at ambient temperature at concentrations adjusted to the expected equilibrium constants and at emission wavelength values given in the supplementary material. All solutions were deoxygenated by purging with nitrogen for 5 min before measurements. The excitation wavelength for ANS and TNS was 375 or 366 nm, respectively.

NMR spectra were recorded on a Bruker AM400/Aspect 3000 system at 295 ± 2 K and 400 MHz. The ROESY spectra were measured without degassing by using the pulse sequence proposed by Griesinger and Ernst.[16] A DANTE (12) sequence of 4000 pulses of 12 μs (60°) with a delay of 63 μs was used for spin-locking on the transmitter channel. The spin-locking field was at the beginning approximately 2 kHz and then dropped continuously to 1.6 kHz. Typical measuring conditions were as follows: sweep width 5000 (2500) Hz; data size 2 K/1 K in 2/1 direction, n/3 shifted squared sine bell in both directions, delay between the scans 2 s; 8 or 16 scans.

Calculations were performed for 1:1 complexes as described earlier[10] for the NMR technique. For fluorescence titrations requiring adjustment of the emissions to the change of volumes and concentrations as well as for evaluation of more complicated equilibria, a program CHEMSIM written by R. Kramer for ATARI 1040 was used, which allows curve fitting for multistep equilibria by numerical integration by using a Newton zero search in several dimensions. Due to the alternating use of Simplex and Newton-Raphson procedures[17] the program shows usually fast convergence at the *same* set of parameters (equilibrium constants and "intrinsic" NMR CIS or fluorescence intensities) independent of different starting values for these variables.

Acknowledgment. This work was financially supported by the Deutsche Forschungsgemeinschaft, Bonn, and the Fonds der Chemischen Industrie, Frankfurt. We thank the referees for valuable suggestions.

Supplementary Material Available: Tables I-V with equilibrium constants and spectral parameters and plots 1-6 with graphical representation of selected titrations (8 pages). Ordering information is given on any current masthead page.

(16) Griesinger, C.; Ernst, R. R. *J. Magn. Res.* **1987**, *75*, 261.
(17) Cf.: Press, W. H.; Flannery, B. P.; Teukolsky, S. A.; Vetterling, W. T. *Numerical Recipes*; Cambridge University Press: Cambridge, 1986; pp 254, 269, 289, 312ff.

Fluorescence Decay Kinetics of the Tryptophyl Residues of Myoglobin Single Crystals†

K. J. Willis, A. G. Szabo,* and D. T. Krajcarski

Contribution from the Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6. *Received August 28, 1990*

Abstract: Picosecond time-resolved fluorescence measurements on protein single crystals are described. Fluorescence spectroscopy is used to probe long-range (20 Å) intramolecular interactions, between tryptophyl residues and heme, in the solution and crystalline states of myoglobin. The fluorescence of single crystals of sperm whale myoglobin decays with double exponential kinetics (τ_1 = 15 ps, τ_2 = 70 ps), while that of crystals of yellow fin tuna myoglobin obeys single exponential kinetics (τ = 24 ps). These data are shown to be consistent with a protein structure, at atomic resolution, that is independent of sample state.

X-ray diffraction can provide high-resolution structural information on proteins in the crystalline state. Spectroscopic techniques including NMR,[1] EPR,[2] Raman,[3] and XAFS[4] have been employed to directly probe differences and/or similarities between crystal and solution protein structures. These techniques generally only report on the immediate environment of an atom or bond. Fluorescence spectroscopy has the advantages of high sensitivity and that long-range interactions can potentially be probed. Time-resolved fluorescence measurements on proteins in solution have provided considerable information on protein structure, function, and dynamics.[5] Clearly it would be of interest to study proteins in the crystalline state with this technique.[6]

The individual tryptophyl residues of myoglobin in solution exhibit well-characterized monoexponential decay kinetics with picosecond lifetimes.[7] The simple decay kinetics and short lifetimes are a consequence of the highly efficient energy transfer from the tryptophyl residues to the heme group.[7,8] A further consequence of the efficient energy transfer is that the fluorescence

† Issued as N.R.C.C. Publication No. 31932.

(1) Rothgeb, T. M.; Oldfield, E. *J. Biol. Chem.* **1981**, *256*, 1432-1446.
(2) Fiamingo, F. G.; Brill, A. S.; Hampton, D. A.; Thorkildsen, R. *Biophys. J.* **1989**, *55*, 67-77.
(3) Morikis, D.; Sage, J. T.; Rizos, A. K.; Champion, P. M. *J. Am. Chem. Soc.* **1988**, *110*, 6341-6342.
(4) Lin, S.-L.; Stern, E. A.; Kalb, A. J.; Zhang, Y. *Biochemistry* **1990**, *29*, 3599-3603.
(5) Beechem, J. M.; Brand, L. *Annu. Rev. Biochem.* **1985**, *54*, 43-71.
(6) Time-resolved fluorescence measurements on protein powders have been reported. Desie, G.; Boens, N.; DeSchryver, F. C. *Biochemistry* **1986**, *25*, 8301-8308 and references cited therein.
(7) Willis, K. J.; Szabo, A. G.; Zuker, M.; Ridgeway, J.; Alpert, B. *Biochemistry* **1990**, *26*, 5270-5275.
(8) (a) Hochstrasser, R. M.; Negus, D. K. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 4399-4403. (b) Janes, S. M.; Holton, G.; Ascenzi, P.; Brunori, M.; Hochstrasser, R. M. *Biophys. J.* **1987**, *51*, 653-660.

0002-7863/91/1513-2000$02.50/0 Published 1991 by the American Chemical Society

lifetimes are extremely sensitive to structural changes, which alter the relative separation or orientation of the tryptophyl and heme moieties.[7,8] For example, a change in the tryptophyl to heme separation of just 0.2 Å can potentially result in a measurable change (10%) in the observed fluorescence lifetime.[7] The ease of crystallization, availability of high-resolution X-ray structures, and the features mentioned above suggest that myoglobins would be useful for "calibrating" and assessing the practicality of time-resolved fluorescence measurements on single protein crystals.

Experimental Section

Monoclinic crystals (1.5 × 0.5 mm) of sperm whale met myoglobin (SW Mb) were grown at pH 7 (from commercial material).[9] Orthorhombic crystals (1 × 0.2 mm) of yellowfin tuna met myoglobin[10] (YFT Mb) were grown at pH 7.4 from material prepared according to Fosmire and Brown.[11] The crystals were washed extensively with 70% saturated ammonium sulfate (pH 7 or 7.4, as appropriate) prior to use. For fluorescence measurements single crystals were mounted centrally in a front-face cell, which was subsequently flushed with nitrogen and sealed.[12] The cell was described earlier by Eisinger and Flores.[13]

The time-resolved fluorescence measurements were performed using time-correlated single-photon counting with laser/microchannel plate instrumentation described elsewhere.[14] The vertically polarized exciting light (wavelength 295 nm, unfocussed) was incident on the cell at an angle of 34° to the normal of the cell face, so that specularly reflected light did not reach the emission optics.[13] Emission was detected after passing through a polarizer set at 55° to the vertical, a filter, and a JY H10 monochrometer set at 328 nm (band-pass 4 nm). The filter (glass, 3 mm pathlength) had a transmittance of 0.002% at 295 nm and 29% at 328 nm. The channel width was 1.0 ps, and data were collected in 512 channels. Instrument response functions were determined, using an identical cell, from the Raman scattering of water at 328 nm[15] (full width at half maximum 60 ps). Data analysis procedures and criteria for assessing the quality of the fit have been described earlier.[14]

Steady-state fluorescence measurements were made on a SLM 8000C photon-counting spectrofluorometer in the ratio mode, with polarizers oriented to eliminate anisotropic effects. The excitation wavelength was 280 nm, and scattered light was removed by a 1 mm pathlength glass filter. The spectral resolution was 4 nm.

Results

Single monoclinic crystals of SW Mb were mounted parallel to the cell face such that the flattened {001} crystal face was irradiated. The count rate detected was strongly dependent on the orientation of the crystal. Horizontal orientation (long axis of the crystal orthogonal to the vertically polarized excitation) gave signals up to 2 kHz, while vertical orientation gave at best 0.5 kHz.[16] The emission from single needle-like orthorhombic YFT Mb crystals was weak. Measurable count rates of 0.7 kHz were attained by aligning three single crystals side by side in the horizontal orientation (as defined above). Prolonged irradiation of the crystals caused photodamage as evidenced by a decrease in the observed count rate and an increase in the measured decay time. Repeat measurements indicated that the crystals were stable for an irradiation time of 1000 s.

The results of time-resolved fluorescence measurements on myoglobin crystals are summarized in Table I. YFT Mb crystals showed single exponential decay kinetics with a lifetime of 24 ps. The decay kinetics of SW Mb crystals were found to be biexponential with lifetimes of 15 and 70 ps. To demonstrate our ability to accurately recover such parameters from a single data set, simulated data were generated. A measured instrument response function was convolved with a biexponential decay (τ_1 = 15 ps, τ_2 = 70 ps, $\alpha_1 = \alpha_2 = 0.5$). The peak counts were normalized to 2 × 10^3, and Poisson noise was added. A two-component fit adequately recovered the parameters (τ_1 = 14 ± 1 ps, α_1 = 0.5, τ_2 = 69.5 ± 0.7 ps, α_2 = 0.5). It is important to note that the parameters listed in Table I result from the global analysis of 10 data sets. New crystals were used for each measurement and the irradiation time was limited to a maximum of 500 s. In the case of SW Mb crystals the proportion of the two fluorescence lifetimes was dependent on crystal orientation. Normalized preexponentials (α) for τ_2 of 0.22 (vertical orientation) and 0.57 (horizontal orientation) were determined.[17]

Table I. Myoglobin (met) Emission Decay[a]

protein	τ_1,[b] ps	τ_2, ps	χ^2	SVR
$SW_{(c)}$	14.6 ± 0.2	70.4 ± 0.3	1.09	1.73
$SW_{(s)}$[c]	21.5 ± 0.5	112.5 ± 0.3	1.06	1.87
$YFT_{(c)}$	24.0 ± 0.1		1.09	1.65
$YFT_{(s)}$	33.0 ± 0.2		1.08	1.91

[a] The excitation wavelength was 295 nm and emission was monitored at 328 nm (4 nm band-pass). Stokes Raman scattering at 328 nm from H_2O was used to determine the instrument response function (fwhm 60 ps). Data were collected at 1 ps/channel in 512 channels, at 21 °C. Ten sample data sets were used in the global analysis. Sample decays were collected to 2 × 10^3 (SW) or 1 × 10^3 (YFT) counts in the peak channel. The subscripts (c) and (s) refer to the crystalline and solution states of the protein, respectively. [b] Lifetimes are given with their standard errors as recovered from analysis of a given global data set. The errors are derived from the diagonal elements of the covariance matrix in the nonlinear least-squares analysis. [c] The corresponding solution data, collected under comparable conditions, were taken from ref 7. We note that the results for YFT (s) at pH 7.4 and 6.4 (previous study) were identical.

The intense scattering of light by solid samples can potentially give rise to artifacts in fluorescence lifetime measurements. In our experiment the scattered light signal was minimized by the use of a front face cell at a specific angle,[13] a cut-off filter, and a 4 nm band-pass monochrometer. Replacing the sample cell with a diffuser plate to maximize scattering gave a signal of only 40 Hz. Reanalysis of our data assuming a scattered light contribution (modeled as a δ function of fixed "lifetime" 0.1 ps) did not significantly alter the recovered parameters or fit statistics (YFT Mb: τ_1 = 0.1 ps, τ_2 = 25.5 ± 0.1 ps, χ^2 = 1.06, SVR = 1.79; SW Mb: τ_1 = 0.1 ps, τ_2 = 13 ± 0.4 ps, τ_3 = 71 ± 0.3 ps, χ^2 = 1.08, SVR = 1.79).

The steady-state emission spectrum for a single crystal of SW Mb was measured. The corrected spectrum was superimposable with the corresponding solution spectrum (emission maximum 325 ± 4 nm).

Discussion

For myoglobins in solution, individual tryptophyl residues display single exponential decay kinetics.[7] Since the rate of deactivation of the tryptophyl excited state by energy transfer to heme greatly exceeds that of all other competing processes,[8] the observed fluorescence lifetimes are therefore primarily determined by the rate of energy transfer. Energy transfer calculations, using distances and angles obtained from the X-ray coordinates, accurately predicted the observed solution lifetimes.[7,8]

Therefore, we would expect myoglobin in the crystalline state to exhibit tryptophyl fluorescence decay kinetics very similar to those observed in solution. This was found to be the case (Table I). SW Mb, which has two tryptophyl residues (Trp 14 and Trp 7), and YFT Mb, with a single tryptophyl residue (Trp 14), showed biexponential and monoexponential decay kinetics respectively, in both solution and crystal. Comparison of the solution and crystal data, however, shows that in each case the crystal lifetimes are some 30% shorter.

The shorter lifetimes in the crystal could be explained by structural changes between the two states. For example, the 30% difference in Trp 14 lifetimes of SW Mb and YFT Mb can be

(9) Kendrew, J. C.; Parrish, R. G. *Proc. R. Soc. London A* **1956**, *238*, 305–324.
(10) Kretsinger, R. H. *J. Mol. Biol.* **1968**, *38*, 141–143.
(11) Fosmire, G. J.; Brown, W. D. *Comp. Biochem. Physiol.* **1976**, *55B*, 293–299.
(12) A small volume of 70% saturated ammonium sulfate was added below the crystal to prevent desiccation.
(13) Eisinger, J.; Flores, J. *Anal. Biochem.* **1979**, *94*, 15–21.
(14) Willis, K. J.; Szabo, A. G. *Biochemistry* **1989**, *28*, 4902–4908.
(15) Willis, K. J.; Szabo, A. G.; Krajcarski, D. T. *Photochem. Photobiol.* **1990**, *51*, 375–377.
(16) For comparison, replacing the sample cell with an identical cell containing water gave a signal (Stokes Raman) of 250 Hz. The count rate for D_2O, which has its Stokes Raman signal at shorter wavelengths (318 nm), was 50 Hz. The effective cell path length was 1.2 mm.
(17) These values should be considered approximate due to the low precision in the crystal orientation.

accounted for by the 0.6 Å greater Trp 14 to heme separation in the latter.[7] A more likely explanation, however, is that Trp–heme distances between neighboring myoglobin molecules in the crystal lattice are such that *inter*molecular energy transfer can occur to some extent.[18]

Following the approach of Desie et al.[6] the coordinates of the neighboring molecules surrounding a central molecule in the SW Mb crystal lattice were generated by translations of the crystallographic unit cell (which contains two molecules). In the crystal lattice 10 hemes were found within 50 Å of Trp 7, 13 in the case of Trp 14. The rate constants were calculated for energy transfer from Trp 7 and Trp 14 to each of the hemes within the 50-Å radius with use of the equations given by Hochstrasser and Negus.[8] In this analysis the distances and angles required to evaluate the donor–acceptor separation and orientation factors were obtained from the X-ray coordinates.[19] The other parameters, which include terms for the refractive index, donor radiative lifetime, and overlap integral, were combined as a single constant. A value for this constant was determined from the solution lifetime values[7] and the X-ray coordinates. The expected tryptophyl fluorescence lifetimes in the crystal are given by the reciprocal of the sum of the respective intra- and intermolecular energy transfer rate constants. Calculated values of 16 ps for Trp 14 and 65 ps for Trp 7 are in close agreement with the observed crystal lifetimes of 15 and 70 ps, respectively. The differences between calculated and observed lifetimes correspond to a change in the Trp–heme separation of 0.2 Å,[7] which is comparable to the root-mean-square positional error of 0.3 Å in the X-ray data.[19] Two hemes account for nearly all of the Trp 14 intermolecular energy transfer; in the case of Trp 7 essentially only 4 neighboring hemes are sufficiently close (<30 Å) to compete with the intramolecular heme as acceptors.[20]

The assumption that the myoglobin structure and spectra are unchanged between solution and crystal states is implicit in the above analysis. We have shown that the fluorescence emission spectrum of the myoglobin crystal is identical with that of the solution, similarly no change is found in the absorption spectrum.[21] Our results are therefore consistent with the identity of the solution and crystal structures of SW Mb.

In solution the two fluorescence lifetime components of SW Mb contribute equally.[7] In the crystal, however, the preexponential terms associated with the two lifetimes were dependent on the crystal orientation. This would be expected if the two tryptophyl residues have different orientations within the protein molecule and hence with respect to the crystal axes. Again this result is consistent with the X-ray structure[19] which shows that the two indole rings are virtually orthogonal. In principle the alignment of the tryptophyl residues with respect to the crystal axes could be determined from the fluorescence decay data taken at a series of precise crystal orientations. The analysis becomes complex, however, when there are several molecules in the crystallographic unit cell.

Time-resolved fluorescence measurements on protein crystals afford possibilities other than simply confirming the correctness of existing X-ray structures. By orienting the crystal it is possible to achieve selective excitation of individual tryptophyl residues in multitryptophan protein, facilitating assignment of the decay kinetics. The crystal structure shows the tryptophyl residue in a unique conformation and environment; crystal studies may provide valuable insights on the origin of the complexity of the tryptophyl decay kinetics frequently observed for proteins in solution. Fluorescence anisotropy decay measurements on protein crystals, to probe side-chain and backbone dynamics, will not be complicated by the depolarization due to rotation of the protein molecule.

The results from this study of myoglobins show that accurate time-resolved fluorescence measurements can be performed on single protein crystals. Heme proteins present a severe test of the practicality of such measurements owing to their extremely short fluorescence lifetimes and low quantum yields. It is expected, therefore, that non-heme protein crystals should give readily measurable fluorescence signals which could be useful in the study of their structure and dynamics.

Acknowledgment. Preliminary purification of the YFT Mb was performed by Dr. T. M. Stepanik. We thank R. To for her valuable assistance with protein crystallization and Dr. G. I. Birnbaum for allowing us access to the X-ray coordinates of YFT Mb prior to publication. We also thank Dr. D. Rose for generating the crystal lattice data and helpful discussions.

Registry No. Tryptophan, 73-22-3.

(18) Intermolecular energy transfer has been reported to occur in α-chymotrypsin powders; see ref 6. We note that the trivial reabsorption process is unlikely in heme protein crystals due to the low quantum yield of the tryptophyl residues. Shimura, M.; Ohba, Y. *Jpn. J. Appl. Phys.* **1981**, *9*, 1683–1690.

(19) Tanko, T. *J. Mol. Biol.* **1977**, *110*, 537–568.

(20) The intramolecular Trp–heme separations in SW Mb were 15.0 and 21.5 Å for Trp 14 and Trp 7, respectively. The closest neighboring hemes are generated by translations of the crystallographic unit cell (axes *a*, *b*, *c*) by $-b$ and $-c$.

(21) Eaton, W. A.; Hofrichter, J. *Methods Enzymol.* **1981**, *76*, 175–261.