NUMBER 12  MARCH 1990

LOCAL NEWS:

This newsletter is the second in a series which contain articles written by Facility users. The first article (submitted by Prof. Jane Vanderkooi and Dr. Sandor Papp of the Department of Biophysics) describe transient absorption studies of metal-substituted myoglobins which probe electronic quenching mechanisms in these compounds. The second article (submitted by Prof. Yale Goldman and Jonathan Tanner of the Muscle Research Institute) discuss fluorescence anisotropy experiments on oriented muscle fibers in order to elucidate the chemical mechanism of muscular contraction.

The optical layout of the picosecond transient absorption apparatus is near completion and will soon be available for regular use. If you have any projects which require high quality transient absorption measurements from tens of picoseconds to milliseconds we would like to hear from you.

On page eight of the newsletter is an announcement of our next Facility minisymposium. This year we have chosen to highlight a wider range of laser-based techniques rather than concentrate strictly on ultrafast techniques. If you are interested in attending, please call or write to me.

Dr. Sam Abrash, our temporary replacement for Gary Holtom, has accepted a position in the Chemistry department at the University of Richmond. We wish him well in all his future endeavors. We are in the process of acquiring a new staff laser research specialist. An advertisement describing the qualifications for this position appeared in the March 12th issue of Chemical and Engineering News. If any of our readers know of a qualified candidate, please contact us.

LASERS IN BIOLOGY: NEEDS AND STATUS

Within the past year, several panels have been commissioned by various agencies (most notably DOE and NIH) to examine the status of emerging laser-based technologies and their present and future applications to various disciplines, including the biological sciences. An article mentioning the NIH study and the current funding status of the NIH Regional Resources (of which we are proud to be a member) was recently published (P. Speser and M. Wood, Laser Focus World Feb. 1990, p. 17).

Among the panels’ suggestions for future development and improvement are:

1) A need to understand the dynamics of transition states in chemical reactions- achieving this goal will answer how enzymes work and make enzyme engineering a reality.

2) Polarized dynamic light scattering measurements- to provide information on chemical and conformational fluctuations in biological macromolecules.

3) Short pulse X-ray lasers- for holographic detection of living cells with a decrease in both photodestruction and image blurring.

4) Tunable/ultrashort pulse IR laser systems- to dynamically probe specific portions of biomolecules.

5) Time-resolved energy transfer studies and emission spectroscopy experiments- to understand complex problems such as protein-nucleic acid interactions.

"Future development of pulsed laser systems should be carried out at laser centers that are a part of biochemical and chemical facilities. In this way, advances in technology will be made which address directly the needs of the life-science research community." (Roland F. Hirsch)

Mark Phillips (215)898-3605.
Characterization of excited triplet states of metal substituted myoglobin

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Myoglobin, a monomeric globular protein of molecular weight 18,800 with the function to transport oxygen, is particularly well suited to serve as a model to study the intramolecular motions of proteins. Its structure is known, thereby permitting structural and functional correlations to be made. In this work the triplet state properties of Zn and Pd derivatives of myoglobin and their interactions with quenchers are studied. Both metal derivatives exhibit long triplet state lifetimes at room temperature, but the Zn derivative of myoglobin is five coordinated and is out-of-plane (as is deoxy myoglobin), whereas Pd porphyrin is planar (as is oxy myoglobin). Therefore, quenching reactions of excited state analogues of the two functional conformations of myoglobin can be studied. The high emission yield and rapid intersystem crossing of Pd porphyrin luminescence allows us to compare steady-state intensities and time resolved decay in the time scale from 100 psec to 1 msec.

METHODS

Heme was removed from myoglobin by the method of Teale with minor modifications and was replaced either by Pd-mesoporphyrin, Zn-protoporphyrin or Zn mesoporphyrin. The porphyrin in dimethylformamide was added to the apoprotein dropwise with stirring followed by incubation for 45 min on ice. The ratio of porphyrin to protein was monitored by recording the protein absorption peak at 278 nm to the Soret peak. In order to insure that no aggregation of the porphyrin occurs and that all porphyrin is protein-bound, the porphyrin was added in substoichiometric ratio to the protein.

Samples for transient absorption and phosphorescence measurements were prepared as follows. The protein was dissolved in a buffer containing 0.1 M NaCl, 50 mM Tris-HCl, 0.3% glucose. The buffer was initially degassed under an aspirator and bubbled with argon. A small volume of solution containing glucose oxidase and catalase was added to the samples in the cuvette at a final concentration of 80 nM and 16 nM, respectively. All these procedures were performed under constant flow of argon to exclude air, the cuvettes were then closed with a quartz stopper and sealed with parafilm.

The triplet state spectra were acquired on the RLBL ns transient absorption spectrometer.

RESULTS

Optical properties of excited triplet state metal-substituted myoglobins. In Figure 1 the transient absorption spectra of Zn-MP-myoglobin are presented. The transient absorption spectra of Zn-MP-myoglobin has a maximum around 445
nm and photobleaching at the Soret band (415 nm). The decay kinetics of the transient absorption and the recovery of the photobleaching are nonexponential and the spectra does not have an isosbestic point (not shown).

The transient absorption spectrum of Pd myoglobin is shown in Fig 2. An isosbestic point was observed in the absorption spectra. In addition, the decay profile of phosphorescence could be fit with a single exponential function (not shown); its value was 1.2 msec. The values of the phosphorescence lifetimes are in good agreement with the analysis of the transient absorption decay.

**DISCUSSION**

Replacement of the iron in myoglobin with other metals produces porphyrin derivatives with long fluorescent and phosphorescent lifetimes. Excited states are electronic isomers of the ground state, with both singlet and triplet excited-state molecules being more reactive than the ground state parent. Because these excited states can be "instantaneously" formed by the absorption of light, their decay properties can provide information on the kinetic properties of porphyrin/protein interactions. Zn porphyrin has been used previously to study heme proteins and circular dichroic data suggest that the replacement of the Fe produces no serious tertiary or secondary changes in the myoglobin structure. In this paper we also introduce the use of Pd porphyrin. Pd-MP-myoglobin emits with very strong long-lived luminescence so it can be measured under ordinary steady-state illumination.

The transient absorption spectra of Zn and Pd derivatives of myoglobin, representing the difference spectra of the
singlet ground state and the triplet state, show interesting variations. Within experimental error, the Pd derivative showed a single exponential triplet decay (Fig. 2) and isosbestic points at 409 nm, 498 nm and 518 nm in the transient absorption spectra. These features usually indicate that a single emitting species is present. In contrast, the Zn derivatives do not have an isosbestic point and a shift in the spectra was seen between 100 µsec and 10 msec. The Pd-MP derivative appeared to decay exponentially and with a single emitting species, as indicated by the isosbestic point in the transient absorption spectra. A non-single exponential decay is an indication that more than one species is emitting. The multiple species may exist in the ground state, or be an indication that an excited state reaction is occurring. The Zn porphyrin triplet state is unstable, and in the excited state can undergo distortions in the x-y plane. In addition, the Zn can be fifth-liganded and the complex decay behavior may be due to the special properties of the Zn. We note that the optical transient absorption spectrum of CO dissociated myoglobin does not show shifts between 30 ps and 6.5 ns after photolysis and so in this regard, Pd myoglobin resembles the native protein.

Oxygen quenches the spin-forbidden excited states of both Zn and Pd porphyrins in myoglobin. There is no significant difference in the quenching of the two, suggesting that oxygen is equally accessible to both porphyrins. Because the porphyrin presents such a large target for oxygen quenching, subtle differences in the polypeptide chain in the two metal are likely not to be detected by this method. The value of phosphorescence quenching of porphyrin in myoglobin is about an order of magnitude less than for free porphyrins in solution.

In summary, subtle differences were seen in the electronic relaxation parameters of Pd and Zn substituted myoglobins in that relaxation of the triplet state Pd derivative showed no evidence of structural rearrangement whereas the Zn porphyrin did. No difference was seen in the interaction with oxygen between the excited triplet state Pd or Zn myoglobins showing that oxygen penetrability to the porphyrin is the same for the two analogues of native myoglobin.

REFERENCES

Footnotes: This work was supported by National Institutes of Health grants GM 34448 (JMV), RR 01348 (GRH and CMP) and the University of Pennsylvania.
Investigating the Molecular Mechanism of Muscle Contraction

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INTRODUCTION

How do skeletal muscles shorten and produce force? Since the 1950's, it has been known that the contractile apparatus of muscle consists of two types of filaments -- thin ones containing the protein actin and thick ones containing the protein myosin -- that slide past each other. It is generally believed that the sliding results from interactions between myosin and actin involving myosin cross-bridges, which undergo cycles of attachment to actin, force production, and detachment, when muscle is activated by Ca\(^{2+}\). With each cross-bridge cycle, ATP (adenosine triphosphate) is hydrolyzed to ADP (adenosine diphosphate) and phosphate, providing energy. The precise structural, mechanical, and chemical changes involved in force production however remain a mystery.

One popular suggestion for the protein structural motion leading to force generation is that the myosin cross-bridges rotate while attached to actin filaments; however there is no direct experimental evidence for this model. The original argument in favor of it came from electron microscopy and X-ray diffraction studies, indicating that cross-bridges project from the myosin filaments at right angles in relaxed muscle but at acute angles after removal of ATP\(^1\). Subsequent efforts to detect cross-bridge angle changes during contraction (using paramagnetic and fluorescent probes) have failed for the most part (see Ref 2 for review), except for observations of the orientation of fluorescent iodo-acetamidotetramethyl rhodamine probes attached covalently to the cross-bridge at Cys-707 of the myosin heavy chain. Using absorption dichroism and fluorescence anisotropy measurements, muscle researchers have shown that the rhodamine probes change orientation when cross-bridges, attached to actin, release ADP\(^3,4\). From studies in our laboratory using caged nucleotides (biologically inert compounds that release ATP or ADP upon photolysis by an intense near ultraviolet laser pulse), however, we have concluded that such changes are not primarily associated with the force generation step of the cross-bridge cycle\(^5\).

In order to understand these observations better, we need to know whether the change in orientation of the probes reflects a change in the mobility of the probes relative to the cross-bridge, a local conformational change within the cross-bridge, or in fact a rotation of the entire cross-bridge. To study this question, we have been measuring time-resolved fluorescence anisotropy decay using the time-correlated single-photon counting apparatus (based around a 76 MHz mode locked Nd:YAG/sync-pumped, cavity-dumped dye laser). Skeletal muscle is an elegant system for fluorescence anisotropy studies, since its components are highly ordered: the filaments are oriented along the muscle fiber axis. While in some ways our experiment is a typical one for the regional laser facility, working with single muscle fibers rather than with molecules in solution adds a few wrinkles. Stabilizing the fiber vertically in the cuvette and aligning it with the photodetector are technically challenging, and the interesting geometry demands unique data analysis as well.
METHODS

Fiber Preparation Fibers in bundles dissected from rabbit psoas muscle are labeled by chemically permeabilizing their membranes and incubating them in iodoacetamido-tetramethyl rhodamine; extent and specificity of cross-bridge labeling are assayed biochemically. Most of the labeling is a covalent adduct to the myosin heavy chain at Cys-707 (also termed SH-1). A single fiber, a few millimeters long and about 75 µm in diameter, is then dissected from a fiber bundle under a microscope. Each end is wrapped in an aluminum clip which is placed onto a hook in a wire frame that fits inside a standard fluorescence cuvette. The fiber has been oriented vertically in the cuvette in all of our experiments so far, though the horizontal orientation may provide additional information.

Solutions During fluorescence measurements, the permeabilized fibers are placed in various solutions that enter the filament lattice and produce particular states of muscle activity. Relaxing solution, in which myosin cross-bridges do not interact with actin to produce force, contains 25 mM EGTA to minimize free Ca$^{2+}$ and 5 mM MgATP. Rigor solution, in which cross-bridges stay attached to actin as in rigor mortis, lacks ATP. Rigor+ADP solution, presumably mimicking the state of the cross-bridge cycle immediately preceding ADP release, contains 1-2.5 mM ADP and no ATP. Contracting solution, containing ATP and Ca$^{2+}$, in which cross-bridges cycle, has not been used in our fluorescence anisotropy experiments yet, because fibers tend to become disordered when contracting for several minutes. All solutions are adjusted to pH 7.1 and ionic strength 200 mM, and the experiments so far have been performed at room temperature.

Optical Measurements For molecules in solution, no fluorescence anisotropy is expected from horizontally polarized excitation light (when emission is collected along the axis perpendicular to that of the excitation, as usual), because vertically and horizontally polarized emissions are equal by symmetry. However, with probes oriented relative to a vertical muscle fiber, the vertically and horizontally polarized emissions are not equivalent. Therefore, we make measurements with excitation light polarized horizontally as well as vertically. Excitation wavelength is around 550 nm, near the absorption maximum of iodoacetamidotetramethyl rhodamine, and collected emission wavelength is centered near 590 nm. The fiber is imaged through 1 mm slits in the monochromator.

Data Analysis To interpret changes in fluorescence anisotropy in terms of average angle of probe orientation, a standard geometrical model for cross-bridge probes is assumed. In this model, probes subtend a given angle with the fiber axis and are azimuthally distributed randomly about that axis. We are developing methods to interpret the time course of fluorescence anisotropy decay with this model. We note that the probe dipole orientation may differ from the actual cross-bridge orientation.

RESULTS AND CONCLUSIONS

Initially, we performed experiments at a time sweep of about 80 picoseconds per channel. By fitting total fluorescence collected (vertically polarized plus twice horizontally polarized) with a single exponential decay, fluorescence lifetimes approximately 3.5 nanoseconds were
obtained in each of the different bathing solutions. Sums of two exponentials, with lifetimes of 3.8 and 1.3 nanoseconds and relative amplitudes 3.2 to 1, provided better fits to the data, possibly indicating the presence of two populations of probes, one on Cys-707 and one elsewhere.

Limiting fluorescence anisotropy at long time \((r_\infty)\) was higher in rigor+ADP (0.38 to 0.42) than in rigor (0.16 to 0.22), with excitation polarized along the (vertical) fiber axis, indicating that probes are aligned more axially in the presence of ADP. Long-time fluorescence anisotropy was intermediate in the relaxed condition (0.30 to 0.32). The changes are reversible when solutions are changed back and forth. These findings are in agreement with previously measured steady-state fluorescence and absorption dichroism data.

Figure 1. Anisotropy decay curves for probes on muscle fibers in rigor, rigor+ADP and relaxed conditions.

Surprisingly, the fluorescence anisotropy in the rigor condition appeared to show a larger decay from its zero time value than that in the presence of nucleotides (ATP or ADP). The time course of the anisotropy decay was difficult to resolve at the 80 picosecond time scale since the rotational correlation time appeared to be less than 1 nanosecond. Therefore, we repeated the experiment at a faster time sweep, 8 picoseconds per channel (Fig. 1). A relatively large anisotropy decay was obtained in the rigor condition with a rotational correlation time of approximately 70 picoseconds. In two of the four experiments which have used the 8 picosecond time base, the rapid decay was not apparent, possibly due to fiber disorder or detector alignment problems. Confirming experiments will be performed in the near future.

As a basis for comparison, we also studied the fluorescence anisotropy of free iodoacetamidotetramethyl rhodamine in aqueous solution. As expected, the zero time anisotropy for vertically polarized excitation was close to 0.4, and for horizontally polarized excitation there was no anisotropy. The anisotropy decay appeared to be a single exponential with a rotational correlation time of about 140 picoseconds, slower than that observed for probes in the muscle fibers. The rotational correlation time, \(\tau_{\text{rot}}\) (sec), is related to the size of the rotating moiety by the equation:

\[
\tau_{\text{rot}} = \frac{V \eta}{kT}
\]

where \(V\) is the hydrated volume of the rotating moiety (cm\(^3\)), \(\eta\) is the viscosity (poise), \(k\) is the Boltzmann constant, and \(T\) is the absolute temperature.\(^6\) Using the room temperature (\(T= 293K\)) value of the viscosity of water (0.01 poise), the calculated hydrated volume of the rotating moiety is \(5.7 \times 10^{-22}\) cm\(^3\), or 570 Å\(^3\). The value of \(\tau_{\text{rot}}\) for rotations of the cross-bridge (the molecular weight of myosin subfragment 1 is 140,000) would be approximately 50 nanoseconds, assuming the hydrated specific volume of the protein is 0.8 cm\(^3\)/g.

The above calculations suggest that the observed subnanosecond rotation
is too fast to represent motions of a major domain of the protein, and probably represents wobbling of the probe itself relative to the cross-bridge. Since the amplitude of the rotation is larger in the absence of nucleotide, our tentative conclusion is that the probe is more mobile in the absence of nucleotide. Other work has shown that the cross-bridge as a whole is less mobile in the absence of ATP. Even though the nucleotide binding site is thought to be several nanometers away from the probe attachment site, it is not surprising that nucleotide binding has long-range effects on the cross-bridge, since nucleotides have strong influence on cross-bridge binding to actin. If our tentative conclusion is correct, it is consistent with our result using caged nucleotides that nucleotide binding rather than force generation is primarily responsible for the observed change in orientation of the probe. The rhodamine probe is thus useful as a reporter of nucleotide binding to cross-bridges.

It seems unlikely that the change in probe orientation indicates the expected cross-bridge rotation. If the cross-bridge actually does not rotate during the force producing step of its cycle, then the cartoons of muscle contraction usually seen in textbooks will need substantial revision. Since most forms of cell motility (such as cytokinesis and organelle transport) are based on proteins analogous to actin and myosin of muscle, we think these results are very important. Further time-resolved fluorescence anisotropy experiments, including raising the viscosity with glycerol to slow the rotation, are needed to confirm these initial results.

REFERENCES


RECENT USER PRESENTATIONS

Biophysical Society 34th Annual Meeting
(18-22 February, 1990, Baltimore)

Nanosecond transient absorption spectroscopy of coenzyme B$_{12}$: Quantum yields and spectral dynamics
E. Chen-Georgetown University

Reactions of excited triplet states of Pd and Zn myoglobins
C.M. Phillips, J.M. Vanderkooi and S. Papp-Univ. of Pennsylvania

Time-resolved fluorescence studies of different gramicidin conformations using intrinsic tryptophan and lipid bilayer fluorophores
C.D. Stubbs, J. Lombardi and C. Ho-Thomas Jefferson University

Excited-state properties of DNA
S. Georghiou, G. Ge, R. Weidner, S. Zhu and C.-R. Huang-Univ. of Tennessee

Tryptophan dynamics in skeletal myosin rod
Y. Chang and R. Ludescher-Rutgers Univ.

Flash photolysis of caged-calcium for the study of the calcium binding kinetics for the sarcoplasmic reticulum ATPase
L.J. Delong, C.M. Phillips, J.H. Kaplan and J.K. Blasie-University of Pennsylvania
A. Scarpa-Case Western-Reserve Univ.

Direct observations of ligand dynamics in hemoglobin by subpicosecond infrared spectroscopy
P.A. Anfinrud, C.Han and R.M. Hochstrasser-Univ. of Pennsylvania
The University of Pennsylvania
NIH Regional Laser and Biotechnology
Laboratories

announce a minisymposium on

Applications of Lasers in Biomedical Research

Friday, April 27, 1990
10 a.m. - 5 p.m.
Room 105, LRSM Auditorium
33rd and Walnut Streets, Philadelphia, PA

Speakers:

L. Brand, Johns Hopkins University
Fluorescence studies of proteins

R.M. Hochstrasser, University of Penna.
Recent advances in ultrafast infrared spectroscopy

R.J.D. Miller, University of Rochester
Picosecond phase grating studies of hemeprotein dynamics

G.J. Small, Iowa State University
Energy and electron transfer in photosynthetic systems studied by spectral holeburning

T.G. Spiro, Princeton University
Raman studies of hemoproteins

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