XII SCHOOL OF PURE AND APPLIED BIOPHYSICS

The ever changing world of (hemo)globins

Palazzo Franchetti

Venice, January 28 – February 1, 2008

Organised by:

SIBPA - *Società Italiana di Biofisica Pura e Applicata*

IVSLA - *Istituto Veneto di Scienze Lettere ed Arti*

Scientific Coordinators

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- **Cristiano Viappiani** (University of Parma)

Director of the School

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Timetable

	Monday January 28	**Tuesday January 29**	**Wednesday January 30**	**Thursday January 31**	**Friday February 1**
9.30-10.15		Sylvia Dewilde	Beatrice Vallone	Cristiano Viappiani	Joel Friedman
10.15-11.00		William A. Eaton	Paola Dominici	Maurizio Brunori	Dario Estrin
11.00-11.30		*Coffee break*	*Coffee break*	*Coffee break*	
11.30-12.15		William A. Eaton	Beatrice Vallone	Noam Agmon	
12.15-13.00	Martino Bolognesi	Alberto Boffi	Maurizio Brunori	Alfredo Di Nola	
Lunch					
15.30-16.00		Andrea Mozzarelli	Alessandra Pesce	Marco Cammarata	
16.00-17.00	Martino Bolognesi	Sylvia Dewilde	Dario Estrin	Noam Agmon	
17.00-18.00	William A. Eaton	Alberto Boffi	Stefano Bruno	Alfredo Di Nola	

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Joel M. Friedman *20*

Archaeal protoglobin structure suggests novel ligand diffusion paths and heme-reactivity modulation

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The structural adaptability of the globin fold has been highlighted by the recent discovery of 2-on-2 hemoglobins, of neuroglobin and cytoglobin. Protoglobin (Pgb) from *Methanosarcina acetivorans* C2A, a strictly anaerobic methanogenic Archaea, is the latest entry adding new variability and functional complexity to the hemoglobin superfamily. Sequence comparisons indicate that Pgbs, despite their 30-35% larger size, are structurally related to the single chain Hbs (composed of about 150 amino acids, folded into a 3-on-3 α-helical sandwich, 12-16% residue identity to Pgbs), and to the heme-based aerotaxis transducer sensor domain of *Bacillus subtilis* GCS. Pgbs bind O_2, CO, and NO reversibly *in vitro*. Although functional and evolutionary issues are openly debated, Pgb may facilitate O_2 detoxification *in vivo* by promoting electron transfer to O_2, or may act as CO sensor/supplier in methanogenesis. The crystal structure of oxygenated *M. acetivorans* protoglobin has been just recently reported by our groups at 1.3 Å, together with first insight into its ligand binding properties. Two crystal forms studied highlight a protein fold consisting of nine main helices (labelled Z, A, B, C, E, F, G, H, and H'), partly related to those identified in classical globins and in GCS (Zhang and Phillips, 2003). The Z helix precedes the globin-fold conserved A-helix; it is further preceded by 20 amino acids building a N-terminal loop held, in both structures, next to the heme propionates by hydrogen bonds to residues of the E and F helices in the protein α-helical core. We show that, contrary to all known globins, protoglobin-specific loops and the N-terminal extension completely bury the heme within the protein matrix. Access of O_2, CO, and NO to the heme is granted by protoglobin-specific apolar tunnels reaching the heme distal site from locations at the B/G and B/E helix interfaces. Functionally, *M. acetivorans* dimeric protoglobin displays a selectivity ratio for O_2/CO binding to the heme that favours O_2 ligation, and anti-cooperativity in ligand binding. Both properties are exceptional within the hemoglobin superfamily.

*** In collaboration with: University of Antwerp (Belgium), University of Hawaii (US), University of Roma Tre (Italy), University of Roma "Tor Vergata" (Italy), and University of Genova, CNR-IFM (Italy)*

Evolution of allosteric models for hemoglobin

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There are two parts to the hemoglobin problem and its relation to the model of Monod, Wyman, and Changeux (MWC)[1,2]. The one that has aroused the most controversy is concerned with the effect of oxygen binding to one subunit on the oxygen affinity of its neighboring subunits, i.e. the homotropic effect of MWC. The second part of the problem, namely the regulation of oxygen affinity by small molecules binding distant from the heme (the heterotropic effects of MWC), has received less attention even though it has wider applicability to other multi-subunit proteins. Experimental evidence, beginning with the work of Shulman, Hopfield[3], and Edelstein[4], strongly favors the MWC explanation of homotropic effects[5]. Although the kinetic results of Gibson and coworkers seemed inconsistent with the MWC model[6], experiments with improved time resolution showed that including tertiary conformational relaxation prior to the quaternary transition removed the inconsistency[7]. The most convincing results in favor of the MWC explanation of equilibrium homotropic effects are the single crystal and gel experiments of Mozzarelli and coworkers that show almost perfectly non-cooperative oxygen binding to the T quaternary structure[8-10]. The small deviations, inferred from the tetramer-dimer dissociation experiments of Ackers and coworkers[11,12], can be explained by the dimeric cooperon model of Brunori[13]. The partition function of this model is in fact the exact MWC partition function for hemoglobin by recognizing the fact that the symmetrically equivalent binding units are the $\alpha\beta$ dimers.

The role of tertiary conformational changes in equilibrium measurements were made quantitative in the model of Szabo and Karplus based on the Perutz stereochemical mechanism[14-16]. They showed that tertiary conformational changes are necessary to explain the alteration of T-state affinity by heterotropic effectors, not allowed by the original MWC model. The recent discovery by Viappiani, Bettati and coworkers of R-like kinetic behavior of liganded subunits in the T quaternary structure provides the most dramatic evidence for the role of tertiary conformational changes[17]. Their kinetic and equilibrium results are readily explained by a generalization of the MWC model, in which a pre-equilibrium between two functionally-different tertiary, rather than quaternary, conformations plays the central role[18]. This tertiary two-state model retains the basic ideas of MWC - a conformational pre-equilibrium and no direct subunit-subunit interaction as in a sequential model[19,20]- and provides a simple framework for understanding a wide range of experimental results on hemoglobin in solution, crystals, and gels[17, 21].

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Cytoglobin: the toddler of the family.

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In vertebrates, five globin types have been identified so far of which hemoglobin (Hb) and myoglobin (Mb) are the best characterized. Most recently, globin X was discovered in fish and amphibians, which appears to be absent in higher vertebrates. The physiological role of neuroglobin (Ngb) and cytoglobin (Cygb) is only just beginning to emerge.

Cygb, discovered in 2002, is present at low concentration in all tissues investigated so far. It is coded by a single copy gene in mammals, with 3 introns at position B12.2, G7.0 and C-terminally (HC11.2). Human Cygb is composed of 190 amino acid residues, where the protein core region is structurally related to the other vertebrate globins and as such displays a 3 over 3 alpha helical sandwich. The heme iron atom is hexacoordinated in the absence of gaseous ligands, where the heme iron atom coordinates both the distal and proximal histidine. The structure contains a large apolar protein matrix cavity that may provide a heme ligand diffusion pathway. Cygb forms dimmers where the hydrophobic interface is surrounded by residues providing stabilizing hydrogen bonds and electrostatic interactions. Coupled to this, the oxygen affinity is comparable to Mb (p_{50} = 1 torr) and is cooperative.

Despite these different facts already know for Cygb, the physiological role is still unclear. Because of the predominantly presence in connective tissue fibroblasts and related cell types in body organs, such as osteoblasts, chodroblasts and hepatic stellate cells, a role in the massive production of extracellular matrix proteins was suggested. However Cygb is also present in neuronal tissues where it, as such, plays a different role, especially because of the nuclear localization in neuronal cells. In the latter Cygb plays a protective role, being involved in the detoxification of reactive oxygen or nitrogen species or by supplying oxygen to specific enzymatic reactions. More recently Cygb is found to be associated with cancers, which are highly oxidative-stress related.

It is clear that before the differential functions of Cygb in different cell types can be defined, a lot more experimental work needs to be done.

Flavohemoglobins: one structure, many functions ?

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The flavohemoglobin family comprehends a vast collection of chimeric proteins in which a globin domain is fused with a ferredoxin reductase-like domain. The genes encoding for flavohemoglobins are widely represented in bacteria and yeasts although their distribution does not follow a discernible pattern correlated to their possible functions. Strikingly, the active site environments are strongly conserved among the members of the family thus suggesting a similar functional behaviour within different microorganisms. Biophysical investigations have shown that these proteins are unique within heme based enzymes in that the heme iron is capable of efficient turnover in the ferrous state and catalyzes the two electrons reduction of multiple substrates such as organic and inorganic peroxides, nitric oxide, oxygen, c-type cytochromes and several organic dyes. The reductase activity, taken together with notable lipid membrane binding properties, has suggested that flavohemoglobins might be involved in the reduction of lipid hydroperoxides under conditions of oxidative stress within the bacterial cell. As yet, however, no evidence has been found in vivo for a physiological mechanism related to membrane peroxidation thus leaving open space for novel functional hypotheses.

Hemoglobin-based Oxygen Carriers

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Blood transfusion is a generally safe clinical therapy. However, the need for sophisticated blood processing, storage and cross-matching, religious rules preventing the use of human blood in transfusion, public/societal concerns about the safety of blood products, especially viral contaminations, and the increase of blood demand due to the increase of age population have fuelled the search for safe and effective blood substitutes (1-4). During the past 30 years, several candidate blood substitutes have been developed using haemoglobin, the so-called hemoglobin-based oxygen carriers (HBOC). These products are thought to be injected free in the plasma. However, several issues limit their use. These are:

- Hemoglobin production from unsustainable supplies of outdated human (donor) blood or from cow blood, which may be unacceptable to some patients and may transmit disease (e.g. prions).
- Adverse side effects, such as raised blood pressure caused by hemoglobin scavenging of the chemical mediator, nitric oxide (NO), and renal failure due to hemoglobin dimer ultrafiltration.
- Autoxidation of hemoglobin and interaction between the heme component and NO, leading to the loss of hemoglobin oxygen transport and oxidation products interfering with the vascular redox balance.
- Increased oxygen affinity with respect to hemoglobin in red blood cells due to the absence of allosteric effectors in the plasma, and decreased cooperativity due to hemoglobin chemical and genetic modifications.

Only a deep knowledge of hemoglobin function and allosteric regulation (5) allows solving some of these issues, generating HBOCs that better fit the demand of transport and unload oxygen.

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Neuroglobin: clues to mechanism from structure and kinetics

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Neuroglobin (Ngb) is a globular heme protein expressed in the brain of vertebrates which binds oxygen reversibly, with an affinity comparable to myoglobin (Mb) (1). Despite low sequence identity, the overall 3D fold of Ngb and Mb are very similar (2, 3). Ngb is involved in the protection of the brain from ischemic damage. In spite of considerable interest, however, its mechanism is still unclear.

In Ngb the sixth coordination position of the heme iron is occupied by the distal histidine, in the absence of an exogenous ligand. Endogenous ligation has been proposed as a unique mechanism for affinity regulation and ligand discrimination in heme proteins. This peculiarity might be related to the still-unknown mechanism of Ngb. Upon CO binding, the distal histidine retains (by and large) its position, whereas the heme group slides deeper into a preformed crevice, thereby reshaping the large cavity (290 $Å^3$) connecting the distal and proximal heme sides with the bulk (4-5). The heme relocation is accompanied by a significant decrease of structural disorder, especially of the EF loop, which may be the signal whereby Ngb communicates hypoxic conditions. This change unveils a heme-sliding mechanism that relies on the presence of a large cavity. The pre-formed "slide" facilitates the rupture of the sixth coordination bond thereby controlling ligand affinity.

The reaction of reduced deoxy Ngb with O_2 and NO is slow (t~2 s) and ligand concentration independent, since exogenous ligand binding can occur only upon dissociation of the distal His64 from the ferrous heme iron. By contrast, $NgbO_2$ reacts very rapidly with NO yielding metNgb and NO_3^- *via* a heme-bound peroxynitrite intermediate (5). Steady-state amperometric experiments show that Ngb is devoid of O_2-reductase and NO-reductase activities.

We are investigating the kinetics of oxygen binding on site-directed mutants and we have determined the oxygen affinity by means of kinetics measurements, we have also proposed a candidate for the physiological reductase *in vivo*.

We are carrying out EXAFS and XANES measurements that have revealed fast photoreduction and photolysis induced by X-rays that indicate an unique reactivity of Ngb.

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Plant hemoglobins: still searching for a function

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Contributions to the study of plant hemoglobins (Hbs) with emphasis on non-symbiotic Hbs (nsHbs) from *Arabidopsis thaliana* will be described. Plant Hbs fall into two classes based on sequence homology. Most dicotyledonous plants contain hexacoordinate members of both classes, whereas monocots contain only class 1 nsHbs. In legumes class 2 Hbs have evolved into leghemoglobin, and these plants lack a hexacoordinate class 2 Hb. Leghemoglobins are pentacoordinate oxygen transport proteins found in root nodules where they facilitate oxygen transport to symbiotic nitrogen fixing bacteria. A characteristic of nsHbs is a very high affinity for O_2 because of an extraordinarily low O_2 dissociation constant. This observation suggests that nsHbs do not release O_2, and that these proteins may not function as O_2-carriers. Work aimed at the elucidation of nsHbs function suggests that these proteins play roles in plant cells by modulating levels of NO and, directly or indirectly, regulate a number of NO-dependent procceses in the plant cell.
NsHbs are ancestral to leghemoglobins. A careful comparison of nsHbs structural properties to those of leghemoglobin can provide a starting point for understanding the structural changes necessary to convert a nsHb into a pentacoordinate oxygen transporter.

Structural dynamics of proteins.

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Protein structure is endowed with a complex dynamic nature, which rules function and controls activity. The experimental investigations that yield information on protein dynamics are carried out in solution; however, in most cases, the determination of protein structure is carried out by crystallography that relies on the diffraction properties of a large number of molecules, in approximately the same conformation, arranged in a three-dimensional lattice. Myoglobin, maybe the most thoroughly characterized protein, has allowed the formulation of general principles in the field of protein structure–function correlation and, since the late 1990s, it has been possible to obtain directly some insight into the complex dynamic behavior of myoglobin and other proteins by Laue diffraction.
This chapter describes some of the technological features involved in obtaining reliable data by time-resolved Laue crystallography, with subnanosecond time resolution. A synopsis of the more significant findings obtained by laser photolysis of myoglobin-CO crystals is also presented, emphasizing the more general aspects of dynamics relevant to the complex energy landscape of a protein.

The "2/2" (truncated) hemoglobin family

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"Truncated hemoglobins" are small hemoproteins, found in bacteria, higher plants and unicellular eukaryotes, building a distinct phylogenetic group within the globin super-family.

The discovery of protein sequences belonging to the widespread truncated hemoglobin family has been followed in the last few years by extensive analyses of their three-dimensional structures. Truncated hemoglobins can be classified in three main groups, at the light of their overall structural properties. Crystallographic analyses have shown that all the three groups adopt a 2-on-2 α-helical sandwich fold, based on four main α-helices of the classical 3-on-3 α-helical sandwich found in vertebrate and invertebrate globins. Each of the three groups displays sequence and structure specific features. Among these, a protein matrix tunnel system is typical of group I, a Trp residue at the G8 topological site is conserved in groups II and III, and residue TyrB10 is almost invariant in the three groups. Despite sequence variability in the heme distal site region, a strongly intertwined, but varied, network of hydrogen bonds stabilizes the heme ligand in the three protein groups. Fine mechanisms of ligand recognition and stabilization may vary based on group-specific distal site residues and on differing ligand diffusion pathways to the heme. Taken together, the structural considerations here presented underline that "truncated hemoglobins" result from careful editing of the 3-on-3 α-helical globin sandwich fold, rather than from simple "truncation" events. Thus, "2/2Hb" appears the most proper term to concisely address this protein family.

Computer simulation of chemical reactivity of globins

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We present an investigation of the molecular basis of chemical reactivity modulation in globins using computer simulation**.** Hybrid quantum-classical (QM-MM) calculations are applied to explore distal and proximal effects on ligand binding to the heme. Trends in binding energies and in the kinetic constants are illustrated through a number of selected examples including the truncated-N hemoglobin from *Mycobacterium Tuberculosis*, mammalian myoglobin, the hemoglobin from the parasitic nematode *Ascaris lumbricoides*, the oxygen transporter in the root of leguminous plants Leghemoglobin, and the C*erebratulus lacteus* nerve tissue hemoglobin.
We present also an investigation of the interplay between ligand migration and protein dynamics obtained through classical molecular dynamics (MD) techniques in combination with advanced sampling tools. These techniques yield significant information about free energy profiles and possible secondary docking sites. Results for the deoxy and oxy truncated N hemoglobin of *Mycobacterium Tuberculosis*, presented as an illustrative example, suggest that the truncated hemoglobin N has evolved a dual-path mechanism for selective/distinct migration of O_2 and NO to the heme, to achieve efficient NO detoxification.
Finally, we present also an analysis of the molecular basis of hexacoordination in human neuroglobin, which show that protein oxidation through the formation of a disulfide bridge promotes the stabilization of the pentacoordinated species, thus favoring the reactive state and suggesting a O_2 storage function for neuroglobin.

Studies in protein crystals

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Single crystal UV-visible microspectrophotometry can provide insights into the functional properties of a specific conformation selected by the crystal lattice among the several conformations normally accessible to the protein in solution. It is the case of hemoglobin, that can be crystallized in either the T or the R state. As the crystal lattice prevents the quaternary transition, the two conformations can be separately characterized from the point of view of ligand binding in the absence and presence of allosteric effectors. Such studies on T state hemoglobin crystals proved important in supporting the Monod-Wyman-Changeux model. Deeper insights were gained by coupling studies in single crystals with the characterization of hemoglobin in other immobilized systems, particularly in wet silica gels.

Microspectrophotometers are based on the coupling between a high quality optical microscope, equipped with quartz objectives, and a single beam spectrophotometer. Several instruments are currently available for single crystal studies. Some allow collection of X-ray diffraction data and absorption spectra at the same times, whereas others can operate only off-line. Unlike most instruments, the Zeiss microspectrophotometers and the BioCARS system include a polarizer. Linearly polarized light allows for the collection of spectra along the crystallographic axis, thus providing a more accurate characterization of reaction intermediates. It can also provide structural information, particularly regarding the dynamics of the chromophore orientation upon ligand binding.

Single crystal UV-visible microspectrophotometry is also an ideal complement to X-ray crystallography, as it allows the detection of enzymatic reaction intermediates and the determination of their formation rate. These pieces of information are particularly useful for crystallographic studies, as the crystallization conditions and the lattice constraints on the protein might significantly alter its reactivity with respect to the solution. Enzymatic reactivity in the crystal has been characterized for several proteins, particularly pyridoxal phosphate dependent enzymes.

Laser flash photolysis investigations on hexacoordinated hemoglobins

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Laser flash photolysis methods have provided much of the current functional information on hemoglobins. The central Fe atom is capable of reversibly binding diatomic ligands, including O_2, CO, and NO. The Fe-ligand bond is photolabile, and a reactive unligated state can be transiently generated with a pulsed laser. The photodissociated ligands quickly rebind to the heme and the process can be monitored by transient absorbance methods.
The rebinding kinetics is strongly influenced by the structural and dynamic features of the protein.
On the short time scale, the ligand rebinding kinetics reflects protein dynamics and ligand migration within the protein inner cavities. The characterization of these processes was done in the past mainly by low temperature experiments. The use of silica gels to trap proteins allows the characterization of internal ligand dynamics at room temperature.
For several recently discovered proteins, a competitive reaction with an endogenous His ligand reduces the efficiency of the reaction of the gas with the pentacoordinated species, and leads to formation of a hexacoordinated, low spin species. The endogenous ligand is finally replaced by the diatomic gas on longer time scale.
The low efficiency of this process observed for several proteins makes the latter effect difficult to study in laser flash photolysis experiments, and an integrated use of stopped flow methods is necessary.
We demonstrate the potential of this approach, combined with modern numerical analysis, for two non-symbiotic hexacoordinated hemoglobins from *Arabidopsis thaliana*.

Computer simulations of Myoglobin

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The results of our classical ad quantum computer simulations on Myoglobin will be presented.
Classical molecular dynamics simulation is used to study the structural dynamics of this protein and in particular the intramolecular diffusion of the carbon monoxide in the protein matrix. The simulations have been performed both in solution and in the crystal.
Mixed quantum mechanics/molecular dynamics calculations are used to study electronic properties in solution, such as the IR spectrum of the carbon monoxide in the distal pocket, the electronic spectrum of the Heme and the kinetics of the binding/unbinding reaction of the carbon monoxide with the iron atom.

100ps structural dynamics capabilities using synchrotron radiation x-ray sources: An application to the quaternary transition of Human Hemoglobin.

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Grenoble, Fr

Abstract:
Bright and short x-ray flashes extracted from a synchrotron allow the study of the structural dynamics of proteins at medium resolution (~ 10 Ang) with 100ps time resolution. Heme-based proteins, due to their natural light sensitivity and robustness, are particularly suited for such studies. In this contribution I will discuss the basic components of the experiment apparatus and the informations contained in a scattering experiment (of water/protein solution).
To conclude the structural dynamics accompanying the R-to-T quaternary transition of Human Hemoglobin is presented. The main results are:

- An unexpected fast time scale (~1.5us) for the transition
- Absence of any (quaternary) intermediates
- Demonstration of the structural sensitivity of the current apparatus

If time permits, a short introduction of the next generation x-ray sources and the future plans for the data analysis will be given.

A Hierarchy of Functionally Important Relaxations in Myoglobin

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Various protein relaxation modes couple to its activity on different timescales leading to non-exponential kinetics. As a ligand migrates within the protein, these modes can localize at different points along its trajectory. How can one identify these different modes and determine their activation energies? In order to answer this question, we have used a unique combination of solvents, mutations and theoretical approach. We have studied (in collaboration with Joel M. Friedman) geminate CO rebinding to myoglobin for two viscous solvents, trehalose and sol-gel (bathed in 100% glycerol) at several temperatures. Mutations in key distal hemepocket residues were used to eliminate or enhance specific relaxation modes. The time-resolved data were then analyzed with a modified Agmon-Hopfield model providing excellent fits in cases where a single relaxation mode is dominant. Using this approach we determine the relaxation rate constants of specific functionally important modes and their Arrhenius activation energies.

We have found a hierarchy of distal pocket modes controlling the rebinding kinetics. The ``heme access mode" (HAM) is responsible for the major slow-down in rebinding. It is a solvent-coupled cooperative mode which restricts ligand return from the xenon cavities. Bulky side-chains, like those His64 and Trp29 (in the L29W mutant), operate like overdamped pendulums which move over the binding site. They may be either unslaved (His64) or moderately slaved (Trp29) to the solvent. Small side-chain relaxations, most notably of leucines, are revealed in some mutants (V68L, V68A). They are conjectured to facilitate inter-cavity ligand motion. When all relaxations are arrested (H64L in trehalose) we observe pure inhomogeneous kinetics with no temperature dependence, suggesting that proximal relaxation is not a factor on the investigated timescale.

The first lecture will provide a detailed discussion of the physical observations, whereas the second talk will demonstrate how to use our SSDP Windows application (E.B. Krissinel`) to solve the underlying diffusion equation and fit the time dependence of the protein survival probability to obtain its relaxation rate parameter.

References

1. David Dantsker, Uri Samuni, Joel M. Friedman and Noam Agmon. A hierarchy of functionally important relaxations within myoglobin based on solvent effects, mutations and kinetic model. *Biochimica et Biophysica Acta: Proteins and Proteomics*, 1749: 234-251, 2005.
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A protein dynamic state model: an approach to understanding how functionally important protein dynamics are governed by hydration and osmolytes.

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Protein dynamics are essential for protein function. A systematic picture of how dynamics influence reactivity is lacking. A central starting issue is how to organize the complex array of dynamical phenomena into a scheme that permits analysis of functional processes in terms of not only structure and conformation but also specific categories of dynamics. These issues will be addressed by building on several of recent conceptual and technical developments. A new general formalism will be presented and discussed. It is based on the emerging concept being promoted by Frauenfelder and coworkers that protein dynamics can be organized hierarchically into tiers that reflect the degree to which they are coupled or “slaved” to specific solvent motions. This formulism labels and describes functional protein states in terms of not only conformational and molecular details but also different tiers of dynamics and their coupling to molecular motions in the hydration shell and the bulk solvent. As a consequence we refer to the new model as the *protein dynamic state model* in which not only global tertiary/quaternary structure is accounted for but also the dynamics that are operative during the temporal window over which the sample is being probed. The new model will be illustrated though temperature-dependent ligand recombination processes and time resolved spectroscopy that probe dynamics in myoglobins and hemoglobins. Sol-gel encapsulation, glassy matrices and viscous solutions are used to tune the rates and activation energies of different solvent motions. In addition several new techniques will be presented that directly probe the behavior of hydration waters as a function of osmolytes. Results using these new probes of hydration in conjunction with the kinetic studies will be used provide a framework within the context of the protein dynamic state model for understanding how osmolytes modulate protein dynamics both in solution and in matrices such as sol-gels and sugar-derived glasses.