s1.m4.o1 **Protein Function in the Crystalline State.**
Andrea Mozzarelli[a], Stefano Bettati[b] and Stefano Bruno[a], [a]*Department of Biochemistry and Molecular Biology, and* [b]*Department of Public Health, University of Parma, 43100 Parma, Italy. E-mail: biochim@unipr.it*

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The number of known protein 3D structures is constantly increasing, opening new avenues for the understanding of the molecular basis of complex biological processes. However, the determination of protein structures in the crystalline state provides only a static picture and just a hint on dynamics-based protein action. Thus, structure-function correlations derived from functional properties observed in dilute solution. and structures obtained in the crystalline state are somewhat arbitrary. Therefore, it is of paramount relevance to assess for each individual protein which functional properties are associated with the determined structure and which functional properties are maintained in the crystal with respect to solution. This information can be obtained by single crystal polarized absorption microspectrophotometry [1], that allow i) to measure uv-vis spectral properties, ii) to determine ligand kinetic and binding parameters, iii) to characterize ligand-induced changes of conformational distribution and equilibrium distribution of catalytic intermediates, iv) to define the experimental conditions for the isolation and accumulation of metastable species, eventually suitable for x-ray analysis, and v) to assess potential radiation alterations in protein cryocrystallography. Microspectrophotometric measurements have been carried out on single crystals exploiting the chromophoric properties of pyridoxal 5'-phophate-dependent enzymes, flavin-dependent enzymes, NAD^+-dependent enzymes, heme- and copper-containing proteins, and natural or chemically modified reporter group-containing proteins. Microspectrophotometers on line with x-ray sources at either synchrotron facilities or home labs have been developed for a direct assessment of diagnostic spectral features. A major issue, still unsolved in spite of considerable effort, is the synchronized triggering of a reaction throughout all protein sites in the crystal. Photo-activation is the key strategy but was proved to be successful on a few selected proteins, including MbCO, photoactive yellow protein, photosynthetic reaction center and bacteriorodopsin [1,2]. Pre-diffused photochemically activated caged compounds, originally proposed to be of general applicability in protein kinetic crystallography, have been used in a few cases, exhibiting limitations. We will discuss a few cases that emphasise i) striking differences between crystal data interpretation and functional properties determined in the same physical state, as observed for oxygen binding stoichiometry and allostery in T quaternary state hemoglobin crystals, ii) the subtle role of lattice forces in altering protein function in the crystal, as observed in crystals of O-acetylserine sulfhydrilase, and iii) the relevance of measuring functional properties of structurally determined conformations, as observed in crystals of cystathionine beta-synthase, cystine lyase and GABA aminotrasferase.

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s1.m4.o2 **Adding a Fourth Dimension to Protein Structures: Intermediates, Perturbations and Movies.**
B. Vallone[a], D. Bourgeois[b,c], A. Arcovito[c], A. E. Miele[a], G. Sciara[a], F. Schotte[d], M. Wulff[b], P. Anfinrud[d] & M. Brunori[a]. [a]*Dip. Scienze Biochimiche, Univ. "La Sapienza", Piazzale A. Moro 5, 00185 Roma, Italy.* [b]*LCCP, UMR, Grenoble Cedex, France* [c]*ESRF, Grenoble Cedex, France* [d]*Lab. of Chemical Physics, NIDDK, NIH, Bethesda, MD, USA. E-mail: beatrice.vallone@uniroma1.it*

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Determining directly the structure of functional intermediates is a task considered unattainable by crystallography, but recent developments have allowed achieve this goal to address questions about 3D structure and dynamics. Only in a few cases perturbations can be applied to protein crystals rapidly and homogeneously in order to obtain "molecular movies". We used single-bunch Laue diffraction to study with ns time resolution the conformational changes in crystals of a triple mutant of sperm whale myoglobin (L29Y, H64Q, T67R; denoted "Mb-YQR") upon rupture of the Fe-CO bond by laser photolysis. Outstanding crystal quality, high level of photolysis, optimisation of the ESRF ID09 beamline and efficient data processing allowed to obtain complete data sets to 1.55 Å resolution from 3ns to 3ms after photolysis. As already observed for wt Mb, CO dissociation induces an immediate out of plane motion of the as well as bending of the heme pyrrole ring C towards the distal pocket. However, a number of novel features were discovered. Immediately following dissociation, Y29 swings towards the CO binding location to fill the vacant space. Remarkably, the rotation of Q64 to establish a hydrogen bond with Y29 extends to the microsecond timescale, dragging the whole of helix E towards its position in the deoxy state of the protein. On this timescale, other significant motions of residues and water molecules are identified on the distal site whereas a transient, weak occupation of the "xenon 1" cavity is observed on the proximal site, presumably due to CO still trapped in the matrix. Our observation of asynchronous internal motions in Mb may be taken as the first direct evidence for the complex potential energy surface of a protein. The extended dynamics of the globin's conformational changes is in agreement with the idea that the protein populates different conformational substates [1]. The time course of the 3D structural changes indicates that we have unveiled the conformational relaxation of the globin which may begin in the sub-ns time regime with bending of the heme, but extends over several orders of magnitude in time towards μs, consistently with time-resolved spectroscopy [2]. These results advance our understanding of the conformational relaxation dynamics of myoglobin, and provide the first structural evidence of their extended nature in time, as discovered in the past by time resolved spectroscopy. We are currently extending this approach by studying the rebinding structural dynamics of hemoglobin.

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