IUBMB *Life*, 59(8): 1 – 17, August 2007

informa
healthcare

Critical Review

Multiple Strategies for O_2 Transport: From Simplicity to Complexity

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Summary

O_2 carriers (extracellular and intracellular as well as monomeric and multimeric) have evolved over the last billion of years, displaying iron and copper reactive centers; very different O_2 carriers may co-exist in the same organism. Circulating O_2 carriers, faced to the external environment, are responsible for maintaining an adequate delivery of O_2 to tissues and organs almost independently of the environmental O_2 partial pressure. Then, intracellular globins facilitate O_2 transfer to mitochondria sustaining cellular respiration. Here, molecular aspects of multiple strategies evolved for O_2 transport and delivery are examined, from the simplest myoglobin to the most complex giant O_2 carriers and the red blood cell, mostly focusing on the aspects which have been mainly addressed by the so called 'Rome Group'.

IUBMB *Life*, 59: 1 – 17, 2007

Keywords O_2 carrier; myoglobin; neuroglobin; hemoglobin; erythrocruorin; chlorocruorin; hemerythrin; hemocyanin; red blood cell; ligand binding; molecular structure.

Abbreviations Ery, erythrocruorin; Hb, hemoglobin; Hmc, hemocyanin; Hr, hemerythrin; Mb, myoglobin; Ngb, neuroglobin; 2,3-DPG, 2,3-diphospoglycerate; MWC model, Monod-Wyman-Changeux concerted allosteric two-state model.

Received accepted

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ISSN 1521-6543 print/ISSN 1521-6551 online © 2007 IUBMB
DOI: 10.1080/15216540701308424

INTRODUCTION

Studies on the structure-function relationships in respiratory proteins and their physiological implications have been a pioneering pathway for the development of our understanding at the molecular level of how a macromolecule exerts its role, being regulated by environmental factors. In this respect, works by Adair (*1*) and Wyman (*2, 3*) paved the way to a field of investigation which became explosive when Perutz and Kendrew solved for the first time the three-dimensional structure of myoglobin (Mb) (*4*) and hemoglobin (Hb) (*5*).

Due to the joined efforts and ideas of Eraldo Antonini, Maurizio Brunori, and Jeffries Wyman, under the wise supervision of Alessandro Rossi Fanelli, there was a building up in Rome of scientific results which gave significant and important contributions to the understanding of: (i) the structural determinants of heme-reactivity in the so called 'simple' heme-proteins; (ii) thermodynamics and kinetics of the ligand-linked conformational changes which characterize the cooperative behavior of a biological macromolecule, casting light on the complex molecular mechanisms at the basis of the exchange of information between the various subunits within a multi-subunit structure; (iii) the way in which the concepts developed within the framework of tetrameric Hbs may be applied to much more complicated systems such as the so called 'giant' O_2 carriers; and (iv) the complexity of spatial relationships, which regulate the functional features of an integrated system, as represented by the erythrocyte.

Here, the molecular aspects of multiple strategies evolved for O_2 transport and delivery are reviewed, mostly focusing on the aspects which have been mainly addressed by the so called 'Rome Group'.

LESSONS FROM MONOMERIC GLOBINS

Monomeric globins are invaluable model systems for heme reactivity because of their apparent simplicity. In this context, horse heart mini-Mb, *Aplysia limacina* Mb, and murine neuroglobin (Ngb) represent prototypical model systems to highlight protein folding mechanisms, structure-function relationships, heme reactivity, and heme-based ligand recognition coupled to signal transduction pathway(s).

Protein Minimization: Horse Heart Mini-Mb

The discovery that most of eukaryotic genes possess a mosaic structure in which expressed sequences (exons) are separated by non-coding sequences (introns) has led to the fascinating hypothesis that the protein segments encoded by exons may correspond to distinct structural/functional domains playing specific roles inside a protein (*6, 7*). Regarding the exons/introns structure of heme-protein genes and the relationship between an informational module (exon) and a structural module (protein domain) it is now well established that the peptide corresponding to the central exon of Mb and Hb genes (i.e., residues 31–105 while horse heart Mb is made of 153 amino acids) (*8–10*) contains almost all residues necessary for heme-protein recognition, apo- and holo-protein stability, and heme reactivity. The first and the third exons encode sequences devoted to modulate Mb and Hb reactivity (*11–13*). An exception is represented by soybean legHb whose central exon is splitted in two exons (*14*).

Following this line of thought, a proteolytic fragment (*32–139*) of horse heart Mb, called 'mini-Mb', was prepared and deeply characterized (*15–20*). Mini-Mb mimicks closely the protein domain (residues 31–105) encoded by the central exon of the Mb gene; in fact, the first exon-coded domain is completely removed, whilst 34 amino acid residues of the third exon-coded domain remain at the C-terminal side (*16*).

Mini-Mb binds tightly the heme (1:1 stoichiometry) and displays spectral and reactivity features similar to those of native horse heart Mb (*15, 16*). The main difference between mini-Mb and Mb resides in the stability of their oxygenated complex, possibly reflecting the absence of some heme-protein interactions, not strictly required for the maintenance of the overall structure, but necessary to prevent heme-Fe-atom oxidation (*17*). However, O_2 helps mini-Mb to achieve the folding of native Mb, at least as far as the first coordination sphere is concerned (*17*).

Spectroscopic, kinetic, and dynamic investigations indicate that structural and functional features of mini-Mb mostly reflect those of the domain responsible for the core structural arrangement and heme reactivity, while the two terminal fragments (corresponding to the first and third exons) dampen the amplitude of the structural fluctuations, which accompany ligand binding, thus rendering the native protein stable and functionally more efficient (*17–19*).

Apo-mini-Mb disclosed a new scenario in globin folding when examined in parallel with apo-Mb (*20*). The lack of the A helix and part of the B and H helices and of some crucial tertiary interactions in mini-Mb represents an important test and a challenge for the folding pathway of apo-Mb proposed by various authors (*21, 22*), who postulated a crucial nucleation role played by the A, G, and H helices. Therefore, the structural stability of mini-Mb (where a key role seems to be played by hydrophobic interactions; see *19*) indicates that it may represent a valuable model as a folding intermediate of Mb under non-denaturing conditions, suggesting an alternative sequence of events in the acquisition of native folding in mini-Mb which may or may not be shared under some conditions also by Mb (*20*).

More recently, the results and the hypothesis on structure-function relationship in mini-Mb have been confirmed by the expression of the 29–105 fragment of sperm whale Mb (called 'micro-Mb'), which is closely corresponding to the central exon of the Mb gene; thus, this peptide shows a correct folding for heme recognition as well as native Mb (*23*).

Protein minimization opens new insights for the comprehension of the complex relationships between independent domains and the overall structure in proteins. The investigations on how a protein reaches its correct folding and how many alternative folding pathways exist may open new ways to understand molecular evolution of proteins and the processes of misfolding with related pathologies (*24*).

Aplysia limacina *Mb: An Invertebrate Prototype Globin*

A. limacina is a opisthobranch gastropod (known as the sea hare) living in the coastal waters of temperate regions. As in other molluscs, the main organs where Mb is localized are the buccal muscle (radula) and the triturative stomach, where it facilitates O_2 delivery (*25*); remarkably, the primary O_2-carrier in *A. limacina* is hemocyanin (Hmc) (see *26*).

A. limacina Mb serves as a prototype for holo- and apo-heme-protein folding (*27–34*), for globins lacking the heme distal HisE7 residue (*35–39*), and for reactivity of heme-proteins displaying a tetra- and/or penta-coordinate heme-Fe-atom (*40–44*). Furthermore, *A. limacina* Mb can be taken as the prototype of invertebrate monomeric O_2-carrying heme-proteins, in which the conventional globin fold residues and molecular mechanisms have evolved along a different line from that observed for vertebrate heme-proteins, though achieving a comparable affinity for the heme ligands (*39, 45–47*).

Earlier work carried out on *A. limacina* holo-Mb highlighted the fully reversible thermal unfolding transition (*28*) and the accumulation of a transient intermediate in the kinetic folding pathway (*29*). More recently, it has been shown that the folding of *A. limacina* apo-Mb follows the same multi-state pathway of the mammalian globins, populating two partially structured intermediates. Moreover, it was shown that the main folding intermediate of *A. limacina* globin corresponds to

the main intermediate of mammalian globins. These results showed that the role of the A, G, and H helices in forming the initial native-like core in the folding of globins (*21*) appears to be evolutionarily conserved, from invertebrates to mammals (*31–33*), and it may be consistent with the conclusion reached in other protein families (*48–50*). On the other hand, this statement is not in contradiction with data observed for apomini-Mb (*20*), since the pathway of folding is not necessarily unique.

Both ferric and ferrous unliganded derivatives of *A. limacina* Mb are tetra-coordinated at pH $\sim$4 and penta-coordinated at pH $\sim$6. The acid-to-neutral transition(s) is reversible and affects markedly the heme-protein reactivity (*41–43, 51*). The high reactivity of ferrous and ferric *A. limacina* Mb towards CO and N_3^-, respectively, at pH $\leq$ 4 appears to reflect the pH-dependent cleavage of the proximal HisF8-N_ε-Fe bond following the protonation of the HisF8-N_ε atom. This induces the displacement of the heme-Fe-atom in the heme plane and this is associated to a large increase of reactivity of the unstrained reaction center (*41–43, 51, 52*). This mechanism is supported by fast CO binding to ferrous penta-coordinate *Chironomus thummi thummi* monomeric erythrocruorin (Ery) displaying a Fe-atom heme plane distance of 0.17 Å, to be compared with that of most penta-coordinate heme-proteins showing low ligand reactivity (e.g., 0.55 Å for sperm whale Mb) (see *43, 47, 51*). Unlike other monomeric globins, the reversible penta-to-tetra-coordinate transition (*i.e.*, the heme reactivity) in *A. limacina* Mb is modulated by anions (*40, 43*).

Within this functional model, the rupture of the fifth axial bond, occurring at low pH and leading to the tetra-coordinate form, allows the heme-Fe-atom to relax in the heme plane in ferrous globins and thus makes the rate of formation of the Fe-ligand bond more favorable by -2 kcal/mol. It is interesting to note that a similar difference in the kinetic free energy barrier has been reported for CO binding to human HbA in the T and the R quaternary conformations (*53*). Although in multimeric Hbs other factors come into play, the heme-Fe-atom has been shown to be closer to the heme plane in the R conformation and therefore such a similarity suggests that -2 kcal/mol is actually the free energy to be spent for such a structural transition (see *43, 51*).

At neutral pH, the reactivity of ferrous *A. limacina* Mb towards diatomic ligands (i.e., O_2 and CO) is similar to that of most ferrous monomeric globins (*46, 47, 54*). In contrast, ferric *A. limacina* Mb displays a very high reactivity towards anionic ligands (e.g., N_3^-) when compared to sperm whale Mb, a prototypical vertebrate monomeric heme-protein (*41, 45, 55–58*). This reflects the absence in ferric *A. limacina* Mb of the water molecule bound at the sixth coordination position of the heme-Fe-atom in most ferric heme-proteins, around neutrality. Therefore, ferric *A. limacina* Mb binds N_3^- showing an unhindered active center (*42*) and without suffering competition with the heme-bound water molecule (which has been shown to represent an important rate-limiting step factor) (see *59*) (Fig. 1).

This functional model reflects the absence of the HisE7 residue at the heme distal site of *A. limacina* Mb (*42*). The replacement of HisE7 with Val in either pig or sperm whale Mb drastically lowers ligand affinity (*46*). The HisE7Val natural mutation in *A. limacina* Mb (Fig. 1) appears to represent the main determinant for the low stability of some heme-ligand complexes (*42*). An array of amino acid substitutions in the heme distal pocket appears to complement, at least in part, the HisE7Val natural mutation in *A. limacina* Mb. Mutations, introduced singly and in combination at residues Lys/ArgCD3, HisE7, ThrE10, and ValE11 in pig and sperm whale Mb (*45, 46*) probe the roles of these key heme distal residues in modulating *A. limacina* Mb reactivity. In *A. limacina* Mb, the ArgE10 residue swings into the heme pocket and provides a hydrogen bond(s) to the heme-bound ligand (*37, 38*) (Fig. 1).

Lastly, HisE7 acts as a 'gate' for ligand access to and exit from the heme pocket in globins devoid of an entrance tunnel within the protein matrix (*60, 61*), thus playing a pivotal role in ligand binding and protein dynamics. Site-directed mutagenesis has confirmed the gating role of HisE7 (*45*) and has allowed to postulate a role for the system of cavities where ligands can dock temporarily (*62*). Time-resolved crystallography (*63–65*) and cryo-trapping experiments (*66, 67*) showed how the internal Mb cavity system modulates heme reactivity in a subtle interplay with tertiary and side-chain transitions centered on the topological position E7 (*68*).

In conclusion, heme-protein reactivity is a complex event reflecting the heme-Fe-atom coordination state (penta- or tetra-coordinate), the ligand accessibility to the active center (by heme distal 'gate(s)', i.e., HisE7), and the stabilization of the heme-bound ligand by hydrogen bonding to heme distal residues (e.g., HisE7 and ArgE10) (Fig. 1). Furthermore, different strategies have been evolved by non-vertebrate and vertebrate globins to achieve comparable ligand affinity, representing a case of convergent evolution (*47*).

Neuroglobin: A Hexa-coordinate Stress-responsive Sensor for Signal Transduction in the Brain

Ngb, a model system for reactivity of hexa-coordinate monomeric globins, is expressed in the brain of vertebrates and is involved in the neuroprotection from damage due to hypoxia or ischemia; overexpression of Ngb ameliorates the recovery from stroke in experimental animals (see *69–71*).

Binding of exogenous ligands to Ngb is a complex event which appears to be characterized by: (i) the cleavage of the endogenous sixth ligand bound to the heme-Fe-atom (i.e., HisE7), (ii) the formation of the transient reactive penta-coordinate species, and (iii) the binding of the exogenous ligand (e.g., O_2) to the vacant sixth coordination site of

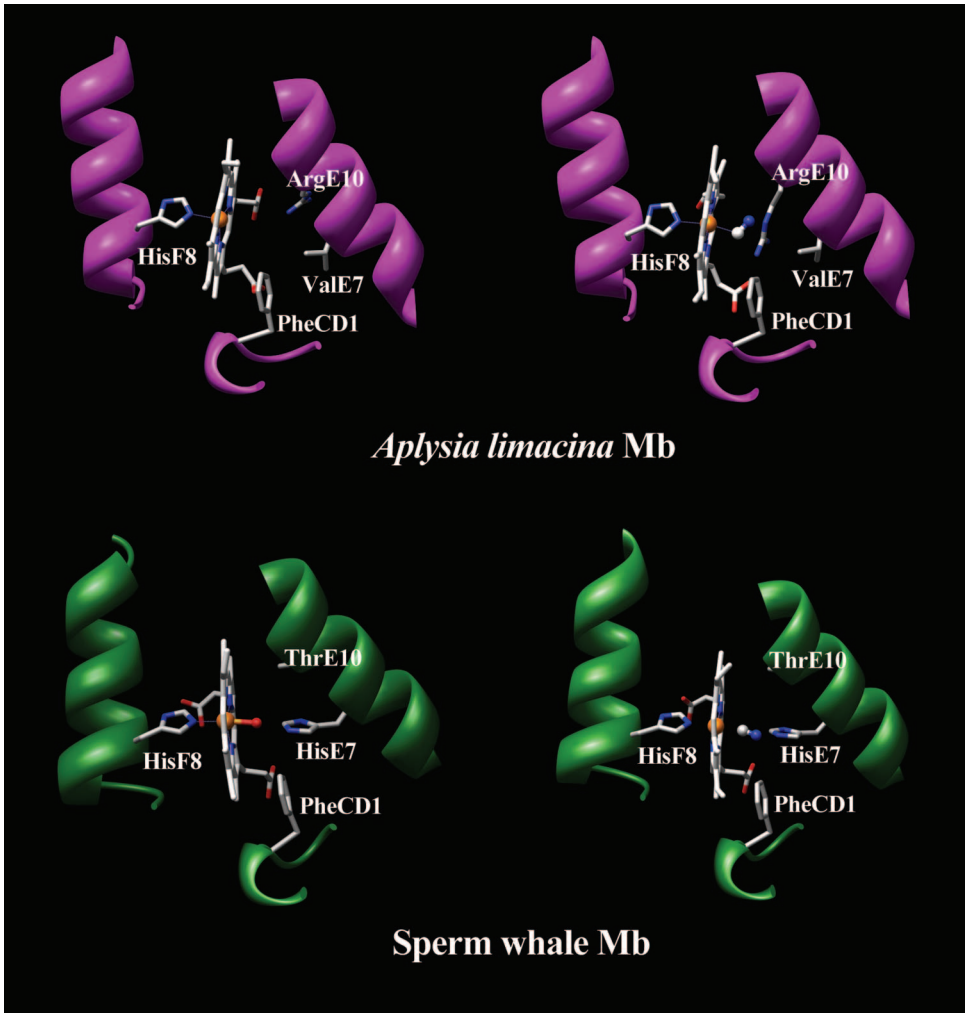

Figure 1. The heme reactive site of ferric *A. limacina* Mb and sperm whale Mb in the absence (left) and presence of cyanide (right). In the absence of exogenous ligands, the ferric heme-Fe-atom of *A. limacina* Mb is penta-coordinated (PDB code, 1MBA; *36*), while sperm whale Mb binds a water molecule (i.e., is hexa-coordinated) (PDB code, 4MBN; *170*), around neutrality. In *A. limacina* Mb, the heme-bound cyanide is stabilized by hydrogen bonding to ArgE10, being the heme distal residue HisE7 replaced by Val (PDB code, 2FAL; *37*). In sperm whale Mb, the heme distal residue HisE7 stabilizes the heme-bound cyanide by hydrogen bonding (PDB code, 1EBC; *171*). All pictures were drawn using the software CHIMERA (http://www.cgl.ucsf.edu/chimera/).

the heme-Fe-atom. As a result of these concurring events, the very high intrinsic affinity of exogenous ligands for the penta-coordinate heme-Fe-atom of Ngb is significantly lowered by internal competition with HisE7, resulting in an apparent affinity of ligands for Ngb closely similar to that of classical penta-coordinate globins (*47, 54, 71–78*).

Ferric hexa-coordinate Ngb shows a huge internal cavity, which connects the heme distal and proximal sides and is in contact with the bulk solvent. The 'entrance' of this cavity is centered around the EF corner, which was found to be characterized by an unusual mobility just like the CD corner (*79, 80*). Binding of CO is associated to a large sliding motion of the heme toward the interior associated to an extensive reorganization of the internal cavity (*81*). Thus, it has been proposed that a role for the huge internal cavity may be to yield space for the heme, thereby facilitating the sliding motion linked to ligand (i.e., CO) binding and the coupled structural changes (such as the change in external mobility of loops and control of access to the tunnel) (*71, 81*) (Fig. 2).

Structural changes accompanying the redox/ligation state have been related to the neuroprotective role of Ngb against hypoxia. Ngb could act as a sensor of the O_2/NO ratio in the cell, possibly regulating the GDP/GTP exchange rate

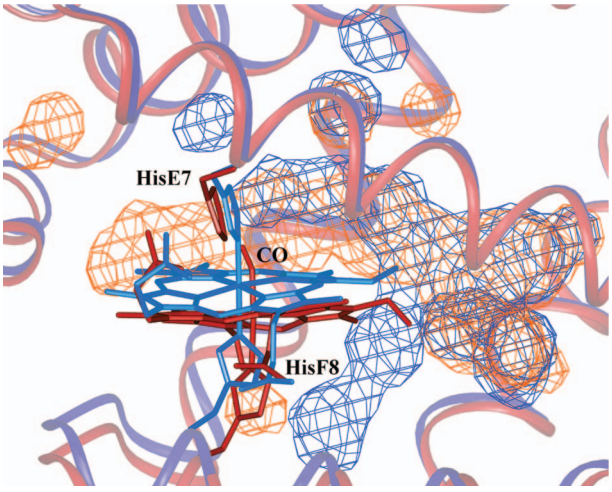

Figure 2. The reactive site of murine ferrous carbonylated Ngb (red) and unliganded ferric Ngb (blue). On CO ligation, the coordination bond with HisE7 (above the heme plane) is broken but imidazole ring moves only slightly, whereas heme tilts and slides toward the right in a pre-existing cavity. This conformational change is associated to (i) a substantial change in the position of the proximal HisF8 residue (seen at bottom) and (ii) the obliteration of the proximal branch of the large cavity (highlighted in blue in ferric Ngb) and its extension on distal side (indicated with orange contour in ferrous carbonylated Ngb) (PDB codes: 1Q1F and 1W92; *80, 81*). Modified from (*71*).

by forming a specific complex with the heterotrimeric $G_{\alpha\beta\gamma}$-protein when oxidized but not when reduced and bound to a gaseous ligand. Ngb oxidation is facilitated under conditions where $[O_2]$ tends to be lower and [NO] higher than normal physiological levels. O_2 binding to Ngb with subsequent very rapid oxidation by NO would have the triple effect of: (i) competing with the direct formation of NgbNO, which is oxidized very slowly by O_2, (ii) reducing free NO, which is harmful to cellular respiration, and (iii) liberating the heterodimeric $G_{\beta\gamma}$-protein and thereby activating the coupled signal transduction pathway, which is protective toward oxidative stress. Ngb:G-protein recognition is based on the homology of the Ngb sequence with that of regulators of G-protein signaling and the corresponding domains of G-protein coupled receptor kinase. However, this appears to be a characteristic of mammalian Ngbs, ferric zebra fish Ngb being devoid of GDI activity. Lastly, Ngb may also protect neurons facilitating directly the degradation and quenching of reactive oxygen species. These possible functions of Ngb demand the demonstration that the brain expresses a significant ferric Ngb reductase activity, which is necessary to restore the reduced state of the heme-protein competent for O_2 binding (see *70, 71, 82*).

LINKED FUNCTIONS AND BINDING POLYNOMIALS: THE THERMODYNAMIC TOOLSET

Mb and mini-Mb often show complex features, rich of information particularly for the understanding of protein folding behavior. Yet, their ligand binding properties are by far the easiest to be described, due to the single binding site for O_2. However, even in the case of moderately larger systems, like tetrameric Hbs (just to avoid mentioning, for the moment, giant systems like Hmcs and hemerythrins (Hrs)), a brand new set of functional features emerges, thanks to the higher number of ligand binding sites influencing each other in many different ways. Therefore, the systematic use of statistical thermodynamics in describing and understanding such complex phenomena allows to account for nonlinear responses to changes in chemical and/or physical variables, so common in such systems, as already illustrated in pioneering works by Wyman and coworkers (*3*).

From this approach a major stream of experimental results and novel theoretical achievements was generated, as in a virtuous loop where modelistic hypotheses called for new experiments, and received from them inspiration for further extension and clarification. Thus, from the concepts of linked functions (*83*), binding potential (*84*) and allosteric conformational transition in the classic form of the Monod-Wyman-Changeux concerted allosteric two-state model (MWC model) (*85*), the cooperative binding of O_2 to Hb, as well as the connection of this process with the binding of H^+ and other ions, found a satisfactory and unified rationale.

The flexibility of the allosteric idea, taken together with the simplicity and euristic power of the MWC model, was soon fully exploited in order to: (i) provide a complete description, under equilibrium conditions, of homotropic (among identical sites) and heterotropic (among different sites) interactions in Hb (*86*); (ii) generalize the approach to the treatment of giant respiratory systems or even enzymes (*87*); and (iii) understand the effects of both physical and chemical variables on protein macromolecules within the same modelistic framework (*88*).

The conceptual elementary basis, underlying such an approach, resides in the idea of representing all macromolecular species of a system in any possible ligation state. This is the binding polynomial, which is a straightforward application of the partition function concept (*83, 89*), for a non dissociating tetrameric Hb its formulation is:

$$P = \Sigma_{i=0,4}\ \beta_i x^i = 1 + \beta_1 x^1 + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4$$

where x is the activity of the X ligand and β_i are the overall binding constants, called Adair constants, for the reaction of adding i ligands to the unliganded macromolecule M (*1*).

Leaving aside the intricacies of the modelistic complications the power of this approach can be easily appreciated just realizing that the first output of most experimental investigations is the fractional saturation (Y) of the protein with the

ligand X. The fractional saturation is immediately provided by the following expression containing P in the denominator and, in the numerator, x times the derivative of P with respect to x:

$$Y = (\beta_1 x + 2\beta_2 x^2 + 3\beta_3 x^3 + 4\beta_4 x^4) / (1 + \beta_1 x^1 + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4)$$

Moreover, if general thermodynamic concepts have been successfully applied almost exclusively to equilibrium conditions, Prigogine and his school extended their application field to the twilight zone between thermodynamics and kinetics, namely steady-state. In the realm of O_2 carriers, a number of interesting examples of such an approach are described at depth in the classical book by Wyman and Gill (*90*).

From the modelistic viewpoint, it is worth mentioning that the kinetic transitions of a system between its two conformations, M1 and M2, were depicted by a functional cycle called the 'turning wheel' (*87*). This model has been used to show that even the catalytic cycle of an enzyme having a single binding site may be described in terms of a cooperative behavior. In this respect, kinetics of CO binding to Hb in the presence of light of different intensities are consistent with a turning wheel model, in which the light of intensity I generates two excited states of the macromolcule, one in its unliganded form, M', and one in its liganded form, M'X (*91*). The rate of excited state generation is dependent on the absorption coefficients of the process, and the light plays the role of a second heterotropic effector. However, studies of CO binding to Mb in the presence of light show that the details of the fast kinetic events involved in photolysis of CO from Mb cannot be included into the simple turning wheel model (*92*). Among the most appealing exploitations of such a model is instead the reciprocal transport of O_2 and CO_2 by the circulating blood or the pH-driven O_2 loading of the swim bladder operated by some fish Hb components (*93*).

TETRAMERIC HEMOGLOBINS: LESSONS ON THE MODULATION OF COOPERATIVITY

Since the formulation of the MWC model (*85*) and the original structural observation by Perutz of the different tertiary and quaternary structures for the unliganded and liganded forms of Hb (*94*), the study of the ligand-linked structural-functional relationships of Hb has become a central topic. Particular attention was addressed toward the relationships between the strain exerted by the bond between the heme-Fe-atom and the proximal HisF8 residue and its spring-like effect toward the interface among the four subunits (especially the $\alpha_1\beta_2$ interface) (see *54*). An additional, and closely related, aspect was the relationships between the ligand-linked release of H^+, O_2 affinity, and quaternary conformational change(s), which had been originally sketched by Perutz as mostly referable to the quaternary-linked variation of the C-terminal residue HisβH24 (*94*). Therefore, at the onset of the 1970s it appeared feasible for the first time to attempt a detailed study of the energetics associated to the ligand-linked quaternary structural changes and to clarify how the structural trigger occurs along the ligand binding pathway (*95*).

In this framework, the work on the thermodynamic aspects of cooperativity of O_2 binding to human Hb carried out first by Imai and Yonetani (*96, 97*) and then by Gill and coworkers (*98, 99*) is of paramount importance. Thus, the detailed analysis reported in these works allowed to attempt a quantification of the energetics involved in individual binding steps, raising important questions about the plausibility of the MWC model for the quantitative description of O_2 binding to human Hb. As a matter of fact, these data seem to suggest the existence of manifold energetic levels for the T quaternary state, a possibility already postulated on the basis of pioneering laser photolysis kinetics of oxygenated human Hb (*100*) and compatible with previous temperature-jump observations (*101, 102*). This hypothesis has been also confirmed in the case of CO binding to human Hb, since it was shown that at acid pH values (where cleavage, or severe weakening of the proximal HisF8-Fe bond has been observed for Mb) (*43, 52*) human Hb undergoes the same phenomenon (*103*) with an increase of the rate constant for CO binding up to values typical of the R state, though keeping the T unliganded conformation. This behavior clearly demonstrated that the stereochemistry of the heme proximal pocket and the proximal HisF8-Fe bond is crucial in modulating the structural-functional relationships associated to the quaternary structural changes following CO binding, this is in line with the stereochemical model proposed by Perutz (*94*).

On the other hand, an additional challenge to the original simple formulation stems from detailed analysis of H^+ release, which does not appear to be uniform along the binding pathway (as expected instead for a purely allosteric effector) and mostly observed during the first binding step (*96, 104*). This observation, which is consistent with the possibility of a manifold T quaternary state, lead to the formulation of an extension of the MWC model (*105*), which allowed to account in a quantitative fashion for some of the most problematic challenges for the MWC model (*106, 107*). In particular, this model postulates the existence of a cooperative unit (called 'cooperon'), within which the interaction among active sites is modulated by direct interactions; the original MWC model operates only among different cooperons which form the whole oligomeric protein. Therefore, this model, which implies the existence of a different cooperon structure according to the number of active sites occupied by the ligand, is able to account for tertiary structural differences (nesting) within a given quaternary overall conformation of the oligomeric protein.

A very important contribution to the comprehension of the modulation of allosteric equilibria in Hb has certainly also

come from the very extensive investigation on the two main components of the Hb system of trout *Salmo irideus* (*108*).

One of these components, called trout HbI, displays cooperative O_2 binding curves which are independent of pH and of any heterotropic effector such as 2,3-diphospoglycerate (2,3-DPG) (*109*). The structural basis for the lack of heterotropic interactions has been proposed to rely on the substitution of some key residues involved in the interaction of organic phosphates (*110*) (Fig. 3). Moreover, trout HbI is characterized by a peculiar temperature effect, such that in the T quaternary state O_2 binding shows no temperature dependence (*i.e.*, $\Delta H_T \cong 0$ kJ/mol) (*111*), and CO binding turns out to be endothermic (i.e., $\Delta H_T = 25.1$ kJ/mol) (*112*). Since for both ligands the R state shows an exothermic ligand binding (i.e., $\Delta H_R < 0$ kI/mol) it was possible to measure quantitatively the temperature perturbation of the allosteric equilibrium as a function of ligand saturation (*111, 113*). These observations on the allosteric equilibria were completed by measurements of the geminate CO recombination at different temperatures associated to a detailed analysis of the rates for the quaternary structural change as a function of temperature (*114*). This measurement allowed to quantify for the first time in a direct way the activation enthalpy for both rates of the allosteric equilibrium in the absence of ligands as well as the linear free energy relations for the quaternary conformational change. Thus, the detailed description of the energetics and the rates of all allosteric equilibria rendered possible to establish that the transition state for the quaternary conformational change is more R-like than T-like, leading to the conclusion that at different ligation steps the progressive population of the R state was due almost completely to the increase of the $T \rightarrow R$ rate whereas the $R \rightarrow T$ rate was left essentially unchanged (*114, 115*). As a whole, this investigation was a stringent test for the applicability of the MWC model, which, in the case of trout HbI, turned out to be perfectly suitable to account for all its functional properties. This was further strengthened by the detailed investigation on the O_2 binding properties of oxidation intermediates of trout HbI (*111*), which can be individually isolated (by virtue of the negligible dissociation to dimers of this Hb and the functional similarity for the two types of subunits) (see *109*). Indeed, the O_2 binding parameters of all oxidation intermediates in trout HbI can be perfectly accounted for by the MWC model (*111*).

A different aspect concerns the relevance of the functional studies on another component of the trout Hb system, called trout HbIV, which displays a marked pH (as well as of other heterotropic ligands) dependence of ligand binding properties. Thus, at moderately acid pH values (i.e., <6.5), trout HbIV is incompletely saturated even at atmospheric O_2 pressures, a phenomenon called 'Root effect' (*93, 108*). Detailed investigation on the pH dependence for O_2 and CO binding (*116, 117*) clearly envisaged a more complex behavior than for trout HbI. Thus, a marked pH dependence of the ligand binding isotherms is accompanied by a dramatic pH dependence of cooperativity, which underlies a linkage between H^+ and the allosteric equilibrium (Fig. 4). This effect turned out to be so

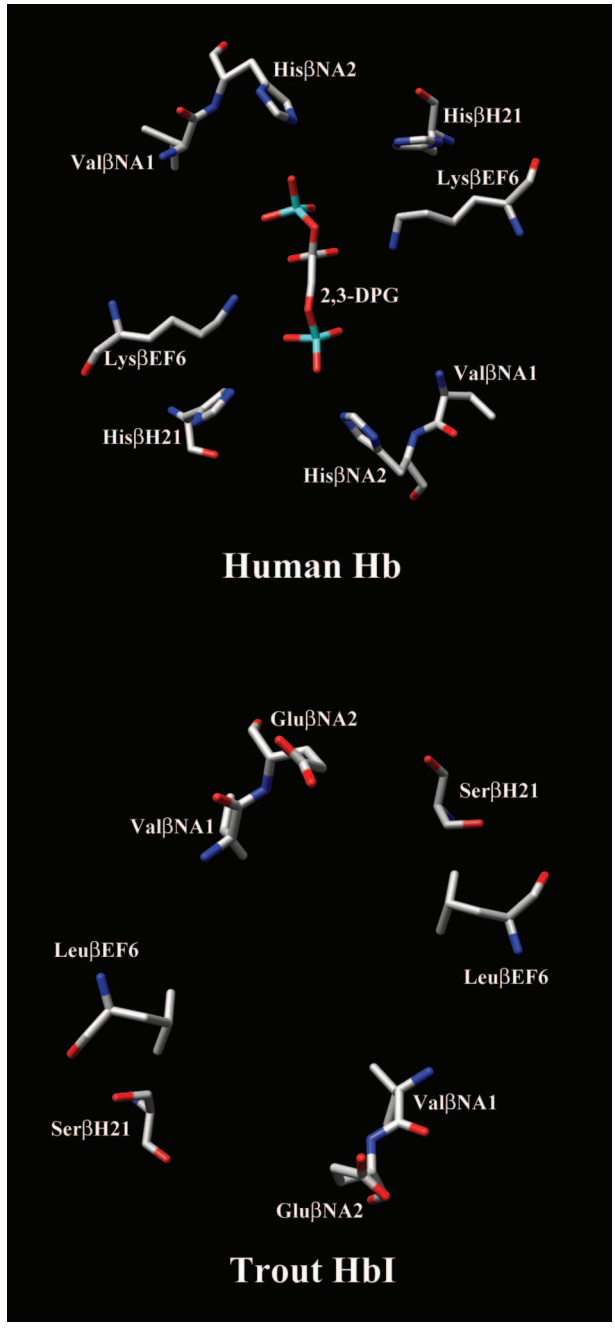

Figure 3. The 2,3-DPG binding site of deoxygenated human Hb (PDB code, 1B86; *172*) and trout HbI (PDB code, 1OUT; *173*). Trout HbI displays cooperative O_2 binding curves which are independent of pH and of any heterotropic effector such as 2,3-DPG (*109*). The lack of heterotropic interactions has been related to the substitution of some key residues involved in the interaction of organic phosphates in human Hb (*110*).

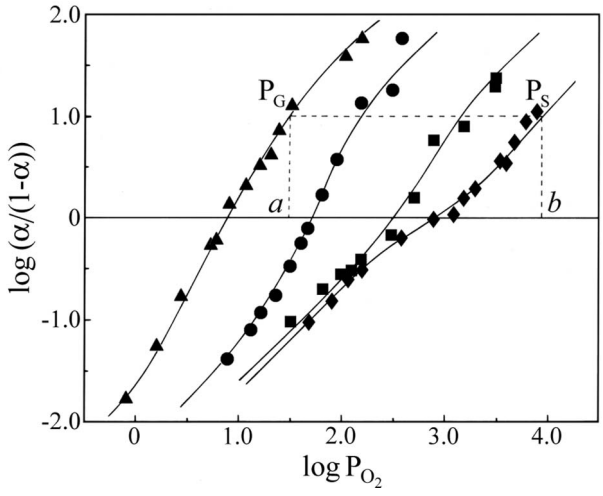

Figure 4. O_2 binding curves of the macromolecular transducer trout HbIV at pH 6.1 (diamonds), 6.7 (squares), 7.15 (circles), and 7.7 (triangles); T = 14°C. The physiological rationale for this marked pH-dependence must be found in the necessity of releasing O_2 from the blood stream also under very high O_2 pressure, such as that present in the swim bladder (*93*). Modified from (*116*).

marked that at pH < 6.5 no quaternary transition occurs and it was possible for the first time to obtain a T-liganded Hb (at least for the CO-bound form), which was characterized by a variation of the spectral properties (*118*). In addition, a pH-dependent variation of the affinity for the T quaternary state can be observed, which is especially marked for O_2 binding, accompanied by a functional heterogeneity for the two types of subunits, which becomes clear-cut at moderately acid pH values (*116*). This behavior cannot be accounted for the simple MWC model and it requires the introduction of additional elements, such as subunit functional heterogeneity and the existence of a nesting of tertiary structures within a given quaternary state (*117*). A marked subunit functional heterogeneity is not infrequent in fish Hbs for both O_2 and CO binding, allowing us to distinguish clearly the behavior of the two chains (*119*); in some cases, it has been possible to fully attribute this subunit functional heterogeneity to variations of the stereochemistry of the proximal bond (*120*).

The physiological rationale for this marked pH-dependence must be found in the necessity of releasing O_2 from the blood stream also under very high O_2 pressure, such as that present in the swim bladder, which is an important element for the regulation of buoyancy (*93*). Thus, trout HbIV acts as a pump, providing O_2 transport and release against a concentration gradient, taking advantage of the marked pH-dependence of the O_2 binding isotherms (Fig. 4). The operation of the pump can be understood on the basis of the following considerations, since reaction rates are fast enough to allow equilibrium to be reached in both gills and swim bladder. If in the gills we have $pH_G = 7.7$ and the blood $pO_2 = a$ (Fig. 4), the O_2 binding behavior of trout blood is represented by the curve P_G, such that the blood takes up O_2 for $pO_2 > a$ (e.g., 1 atm). Going in the swim bladder, where $pO_2 = b$, trout blood could not release O_2 if it keeps the binding behavior of curve P_G. The pump action indeed occurs because in the swim bladder we have $pH_S = 6.1$ and the O_2 binding behavior is represented by the curve P_S (Fig. 4), allowing the trout blood to release O_2 even for $b > pO_2 > a$. In this way, exploiting the marked pH-dependence, trout blood acts as a pump for transferring O_2 from the gills to the swim bladder, i.e., from a region of low pO_2 to one of high partial O_2 pressure. The efficiency of the pump is primarily due to the strong pH dependence of the functional heterogeneity of trout HbIV (Fig. 4), which is considerably greater at the higher saturations associated with high O_2 pressure (*116*).

As a whole, during circulation the blood oscillates between two different states, one as it leaves the capillaries of the gills, the other as it leaves those of the swim bladder. When trout HbIV is acting as a pump, the work of pumping is paid for by the constant return of H^+ from swim bladder, where H^+ concentration is large, to gills, where H^+ concentration is small. The maintenance of the pH gradient, which ensures continuing operation of the system, is due to the metabolism of the fish. It is known that the gas gland, associated to the swim bladder, produces large amounts of lactic acid even in the presence of O_2 (*116, 121*).

Therefore, trout HbIV can be looked upon as a macromolecular transducer, which operates by coupling the concentration gradients of two ligands (i.e., O_2 and H^+), both of which bind to the macromolecule in a linked fashion. The downhill flow of H^+, which is maintained at high concentration in the swim bladder, provides the free energy necessary to pay for the opposite, uphill, flow of O_2; the overall thermodynamic process being of course symmetric with respect to the two ligands (*116*).

The structural basis for the Root effect were proposed by Perutz and Brunori (*122*) as residing in the common replacement of the CysβF9 residue by Ser, whose H-bond with HisβH24 would stabilize the T deoxygenated quaternary conformation, such that the allosteric equilibrium does not stabilize the R form even in the fully liganded species (Fig. 4). However, this is not the only determinant for the Root effect observed in fish and amphibian Hbs, as demonstrated subsequently in a site-directed mutant of human Hb (*123*), where the substitution of CysβF9 by Ser was insufficient to induce an alteration of functional properties similar to those observed in trout HbIV (see *93, 116*).

Starting from the second half of 1980s the possibility of producing site-directed mutants opened a new way of investigating the mechanism of cooperativity, since it was possible to probe directly the functional and structural effect of the substitution of individual aminoacids by different type of residues (*124*); until then, this investigation was only made

possible by natural mutants (*125, 126*). The crucial role of several residues present in the heme distal pocket in determining the intrinsic reactivity toward ligands was elucidated for the different subunits (*127*), putting in evidence that the steric hindrance of the ValE11 residue and the polarity of the HisE7 residue are critical for a fine tuning of ligand binding to human Hb. However, most of these studies focused on the modulation of reactivity, which is easier to investigate, more than on the modulation of cooperativity and allosteric interactions, in particular at the $\alpha_1\beta_2$ interface, which is known to be the central region for the quaternary switch (*128*). In particular, the contacts between the αFG and the βC regions have been proposed to play the role of a 'flexible joint' for the quaternary-linked relative motion of the two subunits, whereas the contacts between the αC and the βFG regions seem to play more the role of the 'switch' for the same structural movement. Therefore, this appears as an important region for the investigation of the role of different amino acids on the allosteric structural change. As a matter of fact, this asymmetric contribution of the two interface regions is demonstrated by the observation that the substitution of ThrαC3 by Trp at the 'switch region' alters the allosteric mechanism, greatly stabilizing the tetrameric assembly in the R liganded structure and keeping a normal cooperativity, while the substitution of TrpβC3 by Thr at 'flexible joint' brings about a destabilization of the tetrameric assembly. The double mutant shows almost no cooperativity, suggesting a gross alteration of the communication between the two regions (*129, 130*). In addition, since the functional difference between the two quaternary conformations for O_2 binding mostly resides on the different ligand dissociation rate constants (*102*), cooperativity can be affected also through site-directed mutation of residues present in the heme distal pocket, which alter the quaternary-linked difference. This has been shown to be the case for mutants where LeuB10 has been substituted by Tyr, which is able to form H-bonds with the bound O_2, dramatically decreasing the ligand dissociation rate constant (*131, 132*). The decrease of cooperativity is however observable only because the effect of the substitution is actually observed only for α-chains, bringing about a dramatic functional heterogeneity for the two types of chains especially in the R quaternary state. This leads to propose that cooperativity is modulated in a different fashion for the two types of subunits, being mostly regulated by 'proximal' variations in the α-chains and by 'distal' variations in the β-chains, as previously predicted on the basis of stereochemical considerations (*133*).

EXTRACELLULAR GIANT OXYGEN CARRIERS: LESSONS ON MULTI-DOMAIN COOPERATIVITY

Different strategies have been adopted during evolution to achieve a consistent solution to the vital problem of O_2 supply to tissues for metabolic demands. Hence, giant Hbs (i.e., Erys from Annelida, Mollusca, and Arthropoda; the prosthetic group is the iron protoporphyrin IX), chlorocruorins (found in Polychaeta; the prosthetic group is a derivative of the iron protoporphyrin IX, in which the vinyl group at position 2 is substituted by a formyl group), Hmcs (from Mollusca and Arthropoda; the reactive site contains two copper atoms), and Hrs (present in Sipunculidis, Priapulidis, and Brachiopods; the reactive site contains two iron atoms) differ greatly in the nature of the prosthetic group (Fig. 5) and of the protein moiety (*134, 135*). However, homotropic and heterotropic effects are common and can be described taking into account ligand-linked conformational changes which may have a similar stereochemical basis. Differences in the cooperative character of O_2 binding displayed by tetrameric Hbs, giant Hbs, and Hmcs are quantitative rather than qualitative. Thus, in the case of extremely large respiratory proteins, both Hmcs and Erys, which may contain even more than 100 O_2 binding sites, a very large value of the Hill coefficient (corresponding to the slope of the Hill plot at 50% saturation) is often coupled with a relatively small total interaction free energy determined by the spacing of the asymptotes of the binding curve. An interpretation of the functional data has been based on the following model (*136*): (i) the binding sites of the whole molecule are segregated into constellations that are essentially independent one of another; (ii) within each constellation, the sites may strongly interact via ligand-linked conformational changes; and (iii) the overall cooperativity is the result of the very strong interactions within each constellation of sites and the opposing effect is the result of the heterogeneity of the functional constellations (*105, 137*).

In spite of the intrinsic approximation, this model has provided a consistent description of the equilibrium data and allowed a uniform treatment of results obtained on Erys and Hmcs from different species and with different molecular weights. However, we cannot disregard that a general problem is concerned with the relationships between functional constellations and structural domains. Considering that functional interactions occur in the assembled molecule and are absent in its dissociation products, it has been reasonable to postulate that the regions of the molecule which are more tightly held together are the ones in which cooperative functional effects are operative. However, since a group of subunits acquires allosteric properties only in the whole molecule it is very likely that functional constellations extend over the boundaries that are identifiable by the structural domains (see *138*).

An improved version of this model, applied to analyze O_2 equilibrium data of *Helix pomatia* β-Hmc, took advantage of the 'cooperon concept' for which each functional constellation is composed of smaller functional units called 'cooperons' (*105*). Such a model, although maintaining several features of the MWC model, incorporates the possibility of the coexistence of tertiary- and quaternary-linked heterotropic effects (*139*).

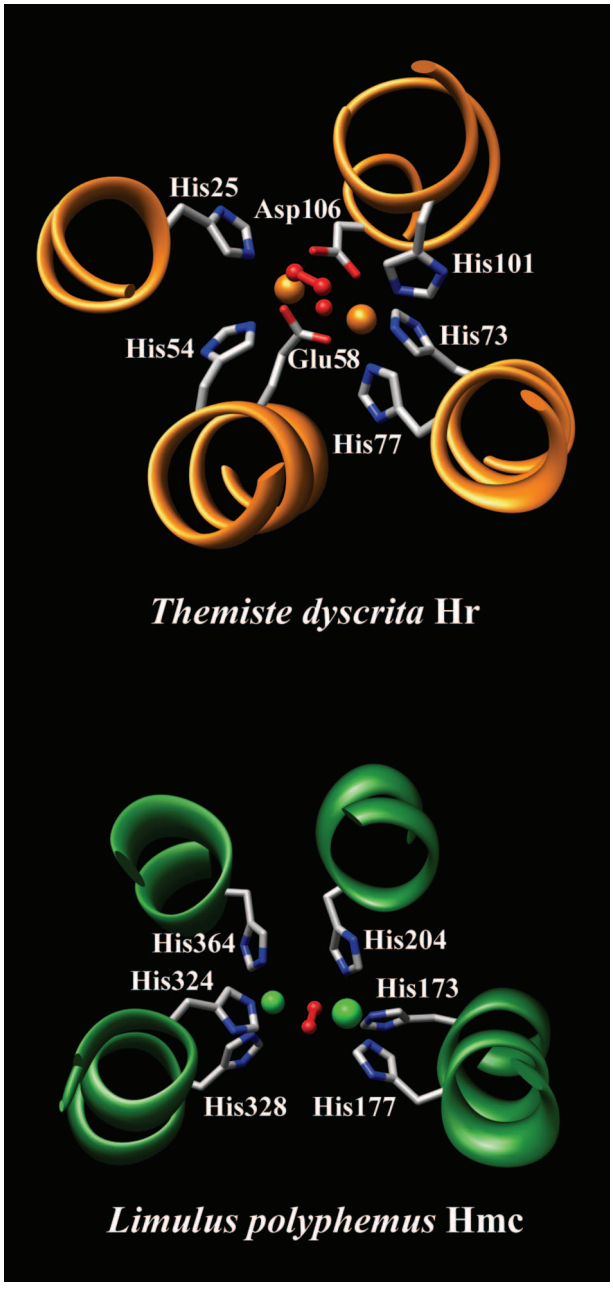

Figure 5. Non-heme reactive site of oxygenated *Themiste dyscrita* Hr (PDB code, 1HMO; *174*), and oxygenated *Limulus polyphemus* Hmc (PDB code, 1OXY; *175*). The binuclear complex of oxygenated *T. dyscrita* Hr retains an oxygen atom bridging the two iron atoms.

Although the molecular architecture of Hmcs, their ligand binding site and the stereochemistry of the binuclear copper center where O_2 is reversibly bound are very different from the corresponding structural features of Hbs and Erys, there are outstanding analogies in the functional properties of these families of O_2 carriers (*105, 134, 135, 140–144*). An example of this analogy is represented by *Panulirus interruptus* Hmc, which exhibits a progressive decrease in O_2 affinity and a parallel loss of cooperativity with decrease in pH, resulting in an apparent loss of the O_2 binding capacity of the protein (*143*). This behavior is strongly reminiscent of the Root effect displayed by some teleost Hbs, such as trout HbIV (*116*). O_2 binding to arthropod Hmcs, such as *P. interruptus* Hmc, is characterized by a $\Delta H_T < \Delta H_R$, underlying that cooperativity in O_2 binding to this Hmc is thermodynamically governed by favorable entropy changes (*144*). Furthermore, like for Hb, the kinetic control of cooperative O_2 binding to Hmcs appears largely determined by the value of the dissociation rate constant while the O_2 combination process is closely similar for the two quaternary states (*141, 144–146*).

The most striking functional difference between Hbs and Hmcs involves the binding of CO, which in the case of Hmcs reacts with a 1:1 stoichiometry (*147*), since, unlike O_2, CO binds only a single cuprous atom Cu(I). Thus, while in Hb or Ery the reaction with CO is associated to homotropic and heterotropic interaction phenomena which are very similar to those observed for the binding of O_2 (*134, 148, 149*), CO binding to Hmcs is non-cooperative and the quaternary-linked Bohr effect is absent. Hence, the cooperative effects in Hmcs are limited to the binding of O_2 and they are strictly related to the bridging properties of this ligand which operates as a 'clamp' between the two copper atoms (Fig. 5). CO, which coordinates with only one of the two metals in a site, has no possibility to trigger the quaternary structural change which is demanded for the allosteric behavior (*150*).

For both molluscan and arthropodal Hmcs, CO binding is largely enthalpy driven, and is associated with a small unfavorable entropy change. Molluscan Hmcs display a CO affinity higher than that of arthropodal Hmcs and the observed differences in equilibrium constant are kinetically reflected in variations for the CO dissociation rate constant (*150*).

A functional heterogeneity in O_2 or CO binding of sites of dissociated molluscan Hmc polypeptide chains was observed in *Helix pomatia* and *Octopus vulgaris* (*151*). From comparison of the O_2/CO affinity ratios (K_{O2}/K_{CO}) of each class it may be suggested that the difference in O_2 affinity of two kinds of binding sites is related to a different local structure of the active sites. Binding and displacement of the two gaseous ligands to Hmc occur by a simple competitive mechanism, although the binding site is structurally complex and the two ligands are bound with different geometries (*135*).

O_2-binding experiments indicate that the allosteric role of Ca^{2+} ions on Hmc functional properties can be played also by lanthanides. Since energy-transfer between lanthanides is only effective over very short distances, the data suggested that some of the cation binding sites of Hmc are clustered (*152*).

Furthermore, the ligand-induced quaternary changes in giant heme-proteins are strictly connected to their complexity. An example of the relationships between structural complexity and allosteric transition is given by chlorocruorin from

polychaete worms (*153–155*). The hierarchical organization of these proteins requires a minimum functional unit of twelve heme sites, coupled by cooperative interactions (*105, 136*). Due to their complexity, quaternary ligand-induced structural changes are quite slow, thus populating long-lived kinetic intermediates (*156*), which may be representative of a transient combination of liganded and unliganded tetramers with the minimum functional dodecameric unit. The overall rate for the quaternary transitions in these larger respiratory proteins was estimated to occur in the second time scale (*156*) (much slower than in smaller heme-proteins, where it occurs in microseconds; see *53*), and it is consistent with their higher complexity. This feature suggests that, although the MWC model can be applied to the description of functional properties of these proteins, more complex phenomena are involved as well in the regulation of their activity.

The very high complexity of these giant O_2 carriers has been further outlined by an intriguing finding that came out from a structural and functional study of *Palinurus elephas* Hmc (*157*). Thus, Hmc from the Mediterranean lobster *P. elephas* has been shown to change in subunit composition in relation to the sex of the animal and the period of the year. The sexual difference, referring to the presence of a specific subunit in the Hmc from female animals, is not linked to any evident functional property of the pigment. On the contrary, the season dependent variations in the subunit composition, which is represented by the appearance of several catodic bands on electropherograms from both male and female animals, produce obvious functional effects; in particular, an increase of the cooperativity of O_2 binding has been consistently found during summer. It is worthwhile to note that the concentration of the pigment is also season-dependent being higher during the winter.

It has been speculated that the additional subunit found in *P. elephas* Hmc could have been related to functions other than respiratory and possibly to other carrying properties (*e.g.*, hormone binding). Although the physiological relevance of the yearly cycle of lobster Hmc is not clear, it seems reasonable to correlate these structural and functional variations with the physiological process of molt, which is known to be accompanied by a large decrease of the Hmc concentration in the hemolymph (*157*).

The main conclusion that is worth outlining is the possible existence, among these giant respiratory proteins, of a sophisticated mechanism(s) of functional modulation which may be achieved not only through the interaction with small molecule and ions but also throughout changes in the ratio of the various subunits (*158*).

KINETICS OF GAS REACTIONS IN ERYTHROCYTES: SINGLE CELL REACTIVITY

This pioneeristic research has been carried out with the aid of a computer-assisted instrument based on the original 'moving condenser' scanning method (*159*). This instrument, combining high resolution microscopy with spectrophotometry, allowed to study intracellular phenomena in living cells and to follow time dependent changes of intracellular components (*160*).

A problem which arises in the case of blood containing multiple Hbs is concerned with the distribution of the various components among red blood cells. In fact, either all the components are represented in each single erythrocyte in the same proportion as in the hemolysate, or else the different Hbs are preferentially distributed and, in the limiting case, each erythrocyte contains only one of the components.

In the case of the blood from trout, which contains four distinct Hb components characterized by different functional and physico-chemical properties, the microspectrophotometric approach has clearly demonstrated that the main Hb component, i.e., trout HbIV, is contained in each red blood cell in a proportion very similar to that of the hemolysate. Thus, taking advantage of the fact that trout HbIV at $pH < 7$ is only partially saturated in air (*116*), a number of absorption spectra taken on several erythrocytes from different preparations has clearly indicated that, in air at $pH \sim 6$, the fractional saturation with O_2 of the different individual erythrocytes corresponds, within experimental error, to that of whole blood. This finding implies unequivocally that each red blood cell contains trout HbIV in a proportion very similar to that of the hemolysate (*161*).

Microspectrophotometric analysis allowed the investigation of the inward-outward gas diffusion in human red blood cells (e.g., CO and O_2), taking advantage of the interaction of intracellular Hb with these ligands and exploiting the photosensitivity of the HbCO complex (Fig. 6). Main results may be summarized as follows. (i) No significant differences in the kinetic properties of several individual cells, and within any single cell among its different spatial regions, were observed. (ii) The approach to the steady-state in the light cannot be accounted for by a single exponential process and its half-time is independent of CO concentration within a factor of two. (iii) The relaxation from the steady-state in the light to the equilibrium in the dark corresponds, under all the conditions explored, to a zero-order process (*160, 162–164*) (Fig. 6).

This body of results showed that combination of CO with Hb in immobile erythrocytes is about 30-fold slower than that measured with Hb in solution and an order of magnitude smaller than that obtained by stopped flow experiments with red cells (*165*). It suggests that this process is rate-limited by a diffusive process, as clearly indicated by the zero-order time course of the observed absorbance change (Fig. 6). This finding has been related to the presence around the erythrocyte of an unstirred layer of fluid, whose thickness has been calculated, on the basis of simple diffusion laws, to be between 6 and 10 μm (*162*). Further, a crucial role was demonstrated to be played also by the intracellular Hb solution, since swelling

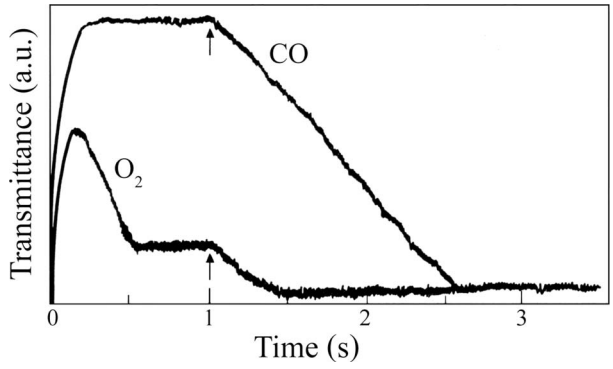

Figure 6. Time course of transmittance changes at 434 nm following irradiation (546 nm) of CO equilibrated human red blood cells. The top trace has been obtained in the absence of O_2, the bottom trace was in the presence of 0.08 mM O_2; CO was present in both experiments at a concentration of 0.1 mM. Upward deflection (at the left) indicates accumulation of deoxygenated Hb, which in the presence of O_2 (bottom trace) disappears to yield HbO_2 according to the following scheme:

$$\text{Hb-CO} + \text{O}_2 \xrightarrow{+h\nu} \text{Hb} + \text{CO} + \text{O}_2 \xrightarrow{+h\nu}$$

$$\text{Hb-O}_2 + \text{CO} \xrightarrow{\text{dark}} \text{Hb-CO} + \text{O}_2$$

The photodissociation beam is kept 'on' from time zero to 1 s and then switched 'off' (arrow). Both traces were recorded at pH 7.4; T = 27°C. Modified from (*164*).

of the erythrocytes under hypo-osmotic conditions brought about a marked increase of the rate of CO uptake (*163*). This clearly demonstrated that ligand diffusion across the intracellular Hb solution was an important rate-limiting step.

In addition, O_2 uptake and release from individual erythrocytes has been measured, exploiting the much higher affinity of human Hb for CO and the low quantum yield for the photodissociation of HbO_2. Thus, experiments carried out in the presence of both O_2 and CO at different ratios allowed us to observe the O_2 uptake kinetics (Fig. 6). Under these conditions the erythrocytes in the dark are fully saturated with CO, but in the presence of a stationary light flux, photodissociation of bound CO occurs and combination with O_2 will follow (Fig. 6). Irradiation of the erythrocyte with a steady flux of light is then followed by distinct phases. (i) The formation of deoxygenated Hb is observed as a transient which is produced with a half time of about 50 ms. (ii) The combination of O_2 occurs with the photoproduct, i.e., deoxygenated Hb, attaining a steady state, corresponding to fully oxygenated Hb. (iii) When the light is turned off, the relaxation in the dark represents the replacement of O_2 by CO and the return to the original absorbance level (*162, 164*).

The analysis of these data allows to conclude that: (i) the time course of O_2 binding, which is sucked into the red blood cell from the surrounding, follows zero-order kinetics indicating that the rate of disappearance of deoxygenated Hb from the volume element under observation is rate-limited by a diffusional process, as for CO uptake; and (ii) the half-time for O_2 binding (about 60 ms) is 600-fold slower than that expected from solution measurements (half time = 0.1 ms) and also much slower than that observed with a population of erythrocytes in rapid mixing experiments (*165*). Moreover, the half-time of O_2 combination is significantly faster by 10 times than that observed in the case of CO (*162, 164*). Since the diffusion coefficient of the two gases is essentially the same, the faster combination with O_2 may be related to the process of facilitated O_2 diffusion (*166*) which is operative from the external boundary to the observation spot.

The photochemical approach described above resulted very useful to follow the dynamics of sickling in erythrocytes containing over 90% hemoglobin S (HbS) (*167*). Hence, CO dissociation induced by light triggers the sickling process which is detected by cell deformation. Experiments have indicated that the rate of the shape change following removal of the ligand is about 4 s. This rate strongly differs from that corresponding to the intracellular polymerization of HbS, which is much faster (*168*), but it is very important information, since it reflects the time required for the transmission of the intracellular molecular events to the shape alteration of the erythrocytes. In addition, it was found that red blood cells in which sickling was induced several times were always deformed along the same axis (*167, 169*). This finding has been related either to an alteration of the red blood cell membrane or to the presence of residual unmelted polymers which may act as templates inducing a preferential direction of sickling.

CONCLUSION

As a whole, these studies on O_2 carriers paved the way to a deeper comprehension of cooperative phenomena in biology and they represent even today basic models for the functional behavior of macromolecules. From the physiological viewpoint, these studies envisage a sort of convergent evolution for a common function. O_2 carriers are responsible for maintaining an adequate delivery of O_2 to tissues and organs almost independently of the environmental O_2 partial pressure. These macromolecules, although characterized by very different structural features, appear to accomplish O_2 transport in a similar fashion, even though they are modulated by somewhat different effectors.

ACKNOWLEDGEMENTS

The authors wish to thank Alberto Boffi, Francesca Cutruzzolà, Giampiero De Sanctis, Fabio Polticelli, Carlo Travaglini-Allocatelli, and Beatrice Vallone for helpful discussions.

REFERENCES

1. Adair, G. S. (1925) The haemoglobin system: the oxygen dissociation curve of haemoglobin. *J. Biol. Chem.* **63**, 529–545.
2. Wyman, J. Jr. (1948) Heme proteins. *Adv. Protein Chem.* **4**, 407–531.
3. Allen, D. W., Guthe, K. F., and Wyman, J. Jr (1950) Further studies on the oxygen equilibrium of haemoglobin. *J. Biol. Chem.* **187**, 393–410.
4. Kendrew, J. C., Dickerson, R. E., Strandberg, B. E., Hart, R. G., Davies, D. R., Phillips, D. C., and Shore, V. C. (1960) Structure of myoglobin: a three-dimensional Fourier synthesis at 2.0 Å resolution. *Nature* **185**, 422–429.
5. Perutz, M. F., Rossmann, M. G., Cullis, A. F., Muirhead, H., Will, G., and North, A. C. T. (1960) Structure of haemoglobin: a three-dimensional Fourier synthesis at 5.5 Å resolution, obtained by X-ray analysis. *Nature* **185**, 416–422.
6. Blake, C. (1978) Do genes-in-pieces imply proteins-in-pieces? *Nature* **273**, 267.
7. Gilbert, W. (1978) Why genes in pieces? *Nature* **271**, 501.
8. Blanchetot, A., Wilson, V., Wood, D., and Jeffreys, A. J. (1983) The seal myoglobin gene: an unusually long globin gene. *Nature* **301**, 732–734.
9. Weller, P., Jeffreys, A. J., Wilson, V., and Blanchetot, A. (1984) Organization of the human myoglobin gene. *EMBO J.* **3**, 439–446.
10. Blanchetot, A., Price, M., and Jeffreys, A. J. (1986) The mouse myoglobin gene: characterisation and sequence comparison with other mammalian myoglobin genes. *Eur. J. Biochem.* **159**, 469–474.
11. Eaton, W. A. (1980) The relationship between coding sequences and function in haemoglobin. *Nature* **284**, 183–185.
12. Gō, M. (1981) Correlation of DNA exonic regions with protein structural units in haemoglobin. *Nature* **291**, 90–92.
13. Lewin, R. (1983) Myoglobin gene is a big surprise: the first analysis of a myoglobin gene reveals some striking similarities and some unexpected differences from hemoglobin genes. *Science* **219**, 1312.
14. Brisson, N., and Verma, D. P. (1982) Soybean leghemoglobin gene family: normal, pseudo, and truncated genes. *Proc. Natl. Acad. Sci. USA* **79**, 4055–4059.
15. De Sanctis, G., Falcioni, G., Giardina, B., Ascoli, F., and Brunori, M. (1986) Mini-myoglobin: preparation and reaction with oxygen and carbon monoxide. *J. Mol. Biol.* **188**, 73–76.
16. De Sanctis, G., Falcioni, G., Giardina, B., Ascoli, F., and Brunori, M. (1988) Mini-myoglobin: the structural significance of haem-ligand interactions. *J. Mol. Biol.* **200**, 725–733.
17. De Sanctis, G., Falcioni, G., Grelloni, F., Desideri, A., Polizio, F., Giardina, B., Ascoli, F., and Brunori, M. (1991) Mini-myoglobin: electron paramagnetic resonance and reversible oxygenation of the cobalt derivative. *J. Mol. Biol.* **222**, 637–643.
18. Di Iorio, E. E., Yu, W., Calonder, C., Winterhalter, K. H., De Sanctis, G., Falcioni, G., Ascoli, F., Giardina, B., and Brunori, M. (1993) Protein dynamics in minimyoglobin: is the central core of myoglobin the conformational domain? *Proc. Natl. Acad. Sci. USA* **90**, 2025–2029.
19. De Sanctis, G., Falcioni, G., Polizio, F., Desideri, A., Giardina, B., Ascoli, F., and Brunori, M. (1994) Mini-myoglobin: native-like folding of the NO-derivative. *Biochim. Biophys. Acta* **1204**, 28–32.
20. De Sanctis, G., Ascoli, F., and Brunori, M. (1994) Folding of apominimyoglobin. *Proc. Natl. Acad. Sci. USA* **91**, 11507–11511.
21. Jennings, P. A., and Wright, P. E. (1993) Formation of a molten globule intermediate early in the kinetic folding pathway of apomyoglobin. *Science* **262**, 892–896.
22. Eliezer, D., Yao, J., Dyson, H. J., and Wright, P. E. (1998) Structural and dynamic characterization of partially folded states of apomyoglobin and implications for protein folding. *Nat. Struct. Biol.* **5**, 148–155.
23. Grandori, R., Schwarzinger, S., and Muller, N. (2000) Cloning, overexpression and characterization of micro-myoglobin, a minimal heme-binding fragment. *Eur. J. Biochem.* **267**, 1168–1172.
24. Panchenko, A. R., Luthey-Schulten, Z., and Wolynes, P. G. (1996) Foldons, protein structural modules, and exons. *Proc. Natl. Acad. Sci. USA* **93**, 2008–2013.
25. Rossi Fanelli, A., and Antonini, E. (1957) A new type of myoglobin isolated and crystallized from the muscles of *Aplysiae*. *Biokhimiya* **22**, 336–344.
26. Lieb, B., Boisguerin, V., Gebauer, W., and Markl, J. (2004) cDNA sequence, protein structure, and evolution of the single hemocyanin from *Aplysia californica*, an opisthobranch gastropod. *J. Mol. Evol.* **59**, 536–545.
27. Brunori, M., Antonini, E., Fasella, P., Wyman, J., Jr. and Rossi Fanelli, A. (1968) Reversible thermal denaturation of *Aplysia myoglobin*. *J. Mol. Biol.* **34**, 497–504.
28. Brunori, M., Giacometti, G. M., Antonini, E., and Wyman, J. Jr. (1972) Denaturation of *Aplysia* myoglobin. Equilibrium study. *J. Mol. Biol.* **63**, 139–152.
29. Giacometti, G. M., Antonini, E., and Brunori, M. (1979) Kinetics of reversible protein denaturation A study on *Aplysia* myoglobin. *Biophys. Chem.* **10**, 119–127.
30. Janes, S. M., Holtom, G., Ascenzi, P., Brunori, M., and Hochstrasser, R. M. (1987) Fluorescence and energy transfer of tryptophans in *Aplysia* myoglobin. *Biophys. J.* **51**, 653–660.
31. Staniforth, R. A., Bigotti, M. G., Cutruzzolà, F., Travaglini-Allocatelli, C., and Brunori, M. (1998) Unfolding of apomyoglobin from *Aplysia limacina*: the effect of salt and pH on the cooperativity of folding. *J. Mol. Biol.* **275**, 143–148.
32. Staniforth, R. A., Giannini, S., Bigotti, M. G., Cutruzzolà, F., Travaglini-Allocatelli, C., and Brunori, M. (2000) A new folding intermediate of apomyoglobin from *Aplysia limacina*: stepwise formation of a molten globule. *J. Mol. Biol.* **297**, 1231–1244.
33. Musto, R., Bigotti, M. G., Travaglini-Allocatelli, C., Brunori, M., and Cutruzzolà, F. (2004) Folding of *Aplysia limacina* apomyoglobin involves an intermediate in common with other evolutionarily distant globins. *Biochemistry* **43**, 230–236.
34. Xia, Z., Nguyen, B. D., Brunori, M., Cutruzzolà, F., and La Mar, G. N. (2005) ^{1}H-NMR study of the effect of temperature through reversible unfolding on the heme pocket molecular structure and magnetic properties of *Aplysia limacina* cyano-metmyoglobin. *Biophys. J.* **89**, 4149–4158.
35. Tentori, L., Vivaldi, G., Carta, S., Marinucci, M., Massa, A., Antonini, E., and Brunori, M. (1973) The amino acid sequence of myoglobin from the mollusc *Aplysia limacina*. *Int. J. Pept. Protein Res.* **5**, 187–200.
36. Bolognesi, M., Onesti, S., Gatti, G., Coda, A., Ascenzi, P., and Brunori, M. (1989) *Aplysia limacina* myoglobin. Crystallographic analysis at 1.6 Å resolution. *J. Mol. Biol.* **205**, 529–544.
37. Conti, E., Moser, C., Rizzi, M., Mattevi, A., Lionetti, C., Coda, A., Ascenzi, P., Brunori, M., and Bolognesi, M. (1993) X-ray crystal structure of ferric *Aplysia limacina* myoglobin in different liganded states. *J. Mol. Biol.* **233**, 498–508.
38. Cutruzzolà, F., Travaglini-Allocatelli, C., Brancaccio, A., and Brunori, M. (1996) *Aplysia limacina* myoglobin cDNA cloning: an alternative mechanism of oxygen stabilization as studied by active-site mutagenesis. *Biochem. J.* **314**, 83–90.
39. Shikama, K., and Matsuoka, A. (2004) Structure-function relationships in unusual nonvertebrate globins. *Crit. Rev. Biochem. Mol. Biol.* **39**, 217–259.
40. Ascenzi, P., Giacometti, G. M., Antonini, E., Rotilio, G., and Brunori, M. (1981) Equilibrium and kinetic evidence for a transition between six- and five-coordinate ferrous heme in the nitric oxide derivative of *Aplysia* myoglobin. *J. Biol. Chem.* **256**, 5383–5386.

41. Giacometti, G. M., Ascenzi, P., Bolognesi, M., and Brunori, M. (1981) Reactivity of ferric *Aplysia* myoglobin towards anionic ligands in the acidic region. Proposal for a structural model. *J. Mol. Biol.* **146**, 363–374.
42. Giacometti, G. M., Ascenzi, P., Brunori, M., Rigatti, G., Giacometti, G., and Bolognesi, M. (1981) Absence of water at the sixth co-ordination site in ferric *Aplysia* myoglobin. *J. Mol. Biol.* **151**, 315–319.
43. Coletta, M., Ascenzi, P., Traylor, T. G., and Brunori, M. (1985) Kinetics of carbon monoxide binding to monomeric hemoproteins. Role of the proximal histidine. *J. Biol. Chem.* **260**, 4151–4155.
44. Rousseau, D. L., Ching, Y. C., Brunori, M., and Giacometti, G. M. (1989) Axial coordination of ferric *Aplysia* myoglobin. *J. Biol. Chem.* **264**, 7878–7881.
45. Brancaccio, A., Cutruzzolà, F., Travaglini-Allocatelli, C., Brunori, M., Smerdon, S. J., Wilkinson, A. J., Dou, Y., Keenan, D., Ikeda-Saito, M., Brantley, R. E. Jr, and Olson, J. S. (1994) Structural factors governing azide and cyanide binding to mammalian metmyoglobins. *J. Biol. Chem.* **269**, 13843–13853.
46. Smerdon, S. J., Krzywda, S., Brzozowski, A. M., Davies, G. J., Wilkinson, A. J., Brancaccio, A., Cutruzzolà, F., Allocatelli, C. T., Brunori, M., Li, T. S., Brantley, R. E. Jr, Carver, T. E., Eich, R. F., Singleton, E., and Olson, J. S. (1995) Interactions among residues CD3, E7, E10, and E11 in myoglobins: attempts to simulate the ligand-binding properties of *Aplysia* myoglobin. *Biochemistry* **34**, 8715–8725.
47. Bolognesi, M., Bordo, D., Rizzi, M., Tarricone, C., and Ascenzi, P. (1997) Nonvertebrate hemoglobins: structural bases for reactivity. *Prog. Biophys. Mol. Biol.* **68**, 29–68.
48. Martinez, J. C., and Serrano, L. (1999) The folding transition state between SH3 domains is conformationally restricted and evolutionarily conserved. *Nat. Struct. Biol.* **6**, 1010–1016.
49. Travaglini-Allocatelli, C., Gianni, S., and Brunori, M. (2004) A common folding mechanism in the cytochrome *c* family. *Trends Biochem. Sci.* **29**, 535–541.
50. Gianni, S., Geierhaas, C. D., Calosci, N., Jemth, P., Vuister, G. W., Travaglini-Allocatelli, C., Vendruscolo, M., and Brunori, M. (2007) A PDZ domain recapitulates a unifying mechanism for protein folding. *Proc. Natl. Acad. Sci. USA*, **104**, 128–133.
51. Traylor, T. G., Deardurff, L. A., Coletta, M., Ascenzi, P., Antonini, E., and Brunori, M. (1983) Reactivity of ferrous heme proteins at low pH. *J. Biol. Chem.* **258**, 12147–12148.
52. Giacometti, G. M., Traylor, T. G., Ascenzi, P., Brunori, M., and Antonini, E. (1977) Reactivity of ferrous myoglobin at low pH. *J. Biol. Chem.* **252**, 7447–7448.
53. Sawicki, C. A., and Gibson, Q. H. (1976) Quaternary conformational changes in human hemoglobin studied by laser photolysis of carboxyhemoglobin. *J. Biol. Chem.* **251**, 1533–1542.
54. Antonini, E., and Brunori, M. (1971) *Hemoglobin and Myoglobin in their Reactions with Ligands* (Neuberger, A., and Tatum, E. L., eds), North Holland Publishing Company, Amsterdam and London.
55. Giacometti, G. M., Da Ros, A., Antonini, E., and Brunori, M. (1975) Equilibrium and kinetics of the reaction of *Aplysia* myoglobin with azide. *Biochemistry* **14**, 1584–1588.
56. Cutruzzolà, F., Travaglini-Allocatelli, C., Ascenzi, P., Bolognesi, M., Sligar, S. G., and Brunori, M. (1991) Control and recognition of anionic ligands in myoglobin. *FEBS Lett.* **282**, 281–284.
57. Mattevi, A., Gatti, G., Coda, A., Rizzi, M., Ascenzi, P., Brunori, M., and Bolognesi, M. (1991) Binding mode of azide to ferric *Aplysia limacina* myoglobin. Crystallographic analysis at 1.9 A resolution. *J. Mol. Recognit.* **4**, 1–6.
58. Leci, E., Brancaccio, A., Cutruzzolà, F., Travaglini-Allocatelli, C., Tarricone, C., Bolognesi, M., Desideri, A., and Ascenzi, P. (1995) Formate binding to ferric wild type and mutant myoglobins thermodynamic and X-ray crystallographic study. *FEBS Lett.* **357**, 227–229.
59. Coletta, M., Angeletti, M., De Sanctis, G., Cerroni, L., Giardina, B., Amiconi, G., and Ascenzi, P. (1996a) Kinetic evidence for the existence of a rate-limiting step in the reaction of ferric hemoproteins with anionic ligands. *Eur. J. Biochem.* **235**, 49–53.
60. Bolognesi, M., Cannillo, E., Ascenzi, P., Giacometti, G. M., Merli, A., and Brunori, M. (1982) Reactivity of ferric *Aplysia* and sperm whale myoglobins towards imidazole. X-ray and binding study. *J. Mol. Biol.* **158**, 305–315.
61. Perutz, M. F. (1989) Myoglobin and haemoglobin: role of distal residues in reactions with haem ligands. *Trends Biochem. Sci.* **14**, 42–44.
62. Scott, E. E., Gibson, Q. H., and Olson, J. S. (2001) Mapping the pathways for O_2 entry into and exit from myoglobin. *J. Biol. Chem.* **276**, 5177–5188.
63. Bourgeois, D., Vallone, B., Schotte, F., Arcovito, A., Miele, A. E., Sciara, G., Wulff, M., Anfinrud, P., and Brunori, M. (2003) Complex landscape of protein structural dynamics unveiled by nanosecond Laue crystallography. *Proc. Natl. Acad. Sci. USA* **100**, 8704–8709.
64. Schotte, F., Lim, M., Jackson, T. A., Smirnov, A. V., Soman, J., Olson, J. S., Phillips, G. N. Jr., Wulff, M., and Anfinrud, P. A. (2003) Watching a protein as it functions with 150-ps time-resolved x-ray crystallography. *Science* **300**, 1944–1947.
65. Bourgeois, D., Vallone, B., Arcovito, A., Sciara, G., Schotte, F., Anfinrud, P. A., and Brunori, M. (2006) Extended subnanosecond structural dynamics of myoglobin revealed by Laue crystallography. *Proc. Natl. Acad. Sci. USA* **103**, 4924–4929.
66. Schlichting, I., Berendzen, J., Phillips, G. N., Jr., and Sweet, R. M. (1994) Crystal structure of photolysed carbonmonoxy-myoglobin. *Nature* **371**, 808–812.
67. Brunori, M., Vallone, B., Cutruzzolà, F., Travaglini-Allocatelli, C., Berendzen, J., Chu, K., Sweet, R. M., and Schlichting, I. (2000) The role of cavities in protein dynamics: crystal structure of a photolytic intermediate of a mutant myoglobin. *Proc. Natl. Acad. Sci. USA* **97**, 2058–2063.
68. Brunori, M., Bourgeois, D., and Vallone, B. (2004) The structural dynamics of myoglobin. *J. Struct. Biol.* **147**, 223–234.
69. Burmester, T., and Hankeln, T. (2004) Neuroglobin: a respiratory protein of the nervous system. *News Physiol. Sci.* **19**, 110–113.
70. Wakasugi, K., Kitatsuji, C., and Morishima, I. (2005) Possible neuroprotective mechanism of human neuroglobin. *Ann. NY Acad. Sci.* **1053**, 220–230.
71. Brunori, M., and Vallone, B. (2006) A globin for the brain. *FASEB J.* **20**, 2192–2197.
72. Perutz, M. F. (1979) Regulation of oxygen affinity of hemoglobin: influence of structure of the globin on the heme iron. *Annu. Rev. Biochem.* **48**, 327–386.
73. Brunori, M., Coletta, M., Ascenzi, P., and Bolognesi, M. (1989) Kinetic control of ligand binding processes in hemoproteins. *J. Mol. Liq.* **47**, 175–193.
74. Pesce, A., Bolognesi, M., Bocedi, A., Ascenzi, P., Dewilde, S., Moens, L., Hankeln, T., and Burmester, T. (2002) Neuroglobin and cytoglobin. Fresh blood for the vertebrate globin family. *EMBO rep.* **3**, 1146–1151.
75. Hamdane, D., Kiger, L., Dewilde, S., Green, B. N., Pesce, A., Uzan, J., Burmester, T., Hankeln, T., Bolognesi, M., Moens, L., and Marden, M. C. (2003) The redox state of the cell regulates the ligand binding affinity of human neuroglobin and cytoglobin. *J. Biol. Chem.* **278**, 51713–51721.

76. de Sanctis, D., Pesce, A., Nardini, M., Bolognesi, M., Bocedi, A., and Ascenzi, P. (2004) Structure-function relationships in the growing hexa-coordinate hemoglobin sub-family. *IUBMB Life* **56**, 643–651. *Erratum* in: (2005) *IUBMB Life* **57**, 459–460.
77. Pesce, A., De Sanctis, D., Nardini, M., Dewilde, S., Moens, L., Hankeln, T., Burmester, T., Ascenzi, P., and Bolognesi, M. (2004) Reversible hexa- to penta-coordination of the heme Fe atom modulates ligand binding properties of neuroglobin and cytoglobin. *IUBMB Life* **56**, 657–664.
78. Fago, A., Mathews, A. J., Dewilde, S., Moens, L., and Brittain, T. (2006) The reactions of neuroglobin with CO: evidence for two forms of the ferrous protein. *J. Inorg. Biochem.* **100**, 1339–1343.
79. Pesce, A., Dewilde, S., Nardini, M., Moens, L., Ascenzi, P., Hankeln, T., Burmester, T., and Bolognesi, M. (2003) Human brain neuroglobin structure reveals a distinct mode of controlling oxygen affinity. *Structure* **11**, 1087–1095.
80. Vallone, B., Nienhaus, K., Brunori, M., and Nienhaus, G. U. (2004) The structure of murine neuroglobin: Novel pathways for ligand migration and binding. *Proteins* **56**, 85–92.
81. Vallone, B., Nienhaus, K., Matthes, A., Brunori, M., and Nienhaus, G. U. (2004) The structure of carbonmonoxy neuroglobin reveals a heme-sliding mechanism for control of ligand affinity. *Proc. Natl. Acad. Sci. USA* **101**, 17351–17356.
82. Brunori, M., Giuffré, A., Nienhaus, K., Nienhaus, G. U., Scandurra, F. M., and Vallone, B. (2005) Neuroglobin, nitric oxide, and oxygen: functional pathways and conformational changes. *Proc. Natl. Acad. Sci. USA* **102**, 8483–8488.
83. Wyman, J. Jr. (1964) Linked functions and reciprocal effects in Hb: a second look. *Adv. Protein Chem.* **19**, 223–286.
84. Wyman, J. Jr. (1965) The binding potential: a neglected linkage concept. *J. Mol. Biol.* **11**, 631–644.
85. Monod, J., Wyman, J., Jr., and Changeux, J.-P. (1965) On the nature of allosteric transitions: a plausible model. *J. Mol. Biol.* **12**, 88–118.
86. Wyman, J. Jr. (1968) Regulation in macromolecules as illustrated by haemoglobin. *Quart. Rev. Biophys.* **1**, 35–80.
87. Wyman, J. Jr. (1975) The turning wheel: a study in steady states. *Proc. Natl. Acad. Sci. USA* **72**, 3983–3987.
88. Wyman, J. Jr. (1984) Linkage graphs: a study in the thermodynamics of macromolecules. *Quart. Rev. Biophys.* **17**, 453–488.
89. Hill, T. (1985) *Cooperativity theory in Biochemistry*, Springer Verlag, NY, USA.
90. Wyman, J., Jr., and Gill, S. J. (1990) *Binding and Linkage*, Univ. Sci. Books, Mill Valley, CA, USA.
91. Brunori, M., Bonaventura, C., Bonaventura, J., Antonini, E., and Wyman, J. Jr. (1972) Carbon monoxide binding by myoglobin and hemoglobin under photodissociating conditions. *Proc. Natl. Acad. Sci. USA* **69**, 868–871.
92. Bonaventura, C., Bonaventura, J., Antonini, E., Brunori, M., and Wyman, J. Jr. (1973) Carbon monoxide binding by simple heme proteins under photodissociating conditions. *Biochemistry* **12**, 3424–3428.
93. Brunori, M. (1975) Molecular adaptation to physiological requirements: the haemoglobin system of trout. *Curr. Topics Cell. Regul.* **9**, 1–39.
94. Perutz, M. F. (1970) Stereochemistry of cooperative effects in Haemoglobin. *Nature* **228**, 726–739.
95. Hopfield, J. J. (1973) Relation between structure, cooperativity and spectra in a model of haemoglobin action. *J. Mol. Biol.* **77**, 207–222.
96. Imai, K., and Yonetani, T. (1975) pH dependence of the Adair constants of human hemoglobin. Non uniform contribution of successive oxygen bindings to the alkaline Bohr effect. *J. Biol. Chem.* **250**, 2227–2231.
97. Imai, K. (1979) Thermodynamic aspects of the cooperativity in four-step oxygenation equilibria of haemoglobin. *J. Mol. Biol.* **133**, 233–247.
98. Gill, S. J., Di Cera, E., Doyle, M. L., Bishop, G. A., and Robert, C. H. (1987) Oxygen binding constants for human hemoglobin tetramers. *Biochemistry* **26**, 3995–4002.
99. Di Cera, E., Robert, C. H., and Gill, S. J. (1987) Allosteric interpretation of the oxygen-binding reaction of human haemoglobin tetramers. *Biochemistry* **26**, 4003–4008.
100. Sawicki, C. A., and Gibson, Q. H. (1977) Properties of the T state of human oxyhemoglobin studied by laser photolysis. *J. Biol. Chem.* **252**, 7538–7547.
101. Brunori, M., and Schuster, T. M. (1969) Kinetic studies of ligand binding to hemoglobin and its isolated subunits by the temperature jump relaxation method. *J. Biol. Chem.* **244**, 4046–4053.
102. Ilgenfritz, G., and Schuster, T. M. (1974) Kinetics of oxygen binding to human haemoglobin. Temperature jump relaxation studies. *J. Biol. Chem.* **249**, 2959–2973.
103. Coletta, M., Ascenzi, P., and Brunori, M. (1988) Kinetic evidence for a role of heme geometry on the modulation of carbon monoxide reactivity in human hemoglobin. *J. Biol. Chem.* **263**, 18286–18289.
104. Di Cera, E., Doyle, M. L., and Gill, S. J. (1988) Alkaline Bohr effect of human haemoglobin A_0. *J. Mol. Biol.* **200**, 593–599.
105. Brunori, M., Coletta, M., and Di Cera, E. (1986) A cooperative model for ligand binding to biological macromolecules as applied to oxygen carriers. *Biophys. Chem.* **23**, 215–222.
106. Smith, F. R., and Ackers, G. K. (1985) Experimental resolution of cooperative free energies of the ten ligation states of human haemoglobin. *Proc. Natl. Acad. Sci. USA* **82**, 5347–5351.
107. Gill, S. J., Robert, C. H., Coletta, M., Di Cera, E., and Brunori, M. (1987) Cooperative free energies for nested allosteric models as applied to human hemoglobin. *Biophys. J.* **50**, 747–752.
108. Binotti, I., Giovenco, S., Giardina, B., Antonini, E., Brunori, M., and Wyman, J. Jr. (1971) Studies on the functional properties of fish hemoglobins, the oxygen equilibrium of the isolated haemoglobin components from trout blood. *Arch. Biochem. Biophys.* **142**, 274–280.
109. Brunori, M., Giardina, B., Chiancone, E., Spagnuolo, C., Binotti, I., and Antonini, E. (1973) Studies on the properties of fish hemoglobins. Molecular properties and interaction with third components of the isolated hemoglobins from trout (*Salmo irideus*). *Eur. J. Biochem.* **39**, 563–570.
110. Barra, D., Bossa, F., and Brunori, M. (1981) Structure of binding sites for heterotropic effectors in fish hemoglobins. *Nature* **293**, 587–588.
111. Colosimo, A., Coletta, M., Falcioni, G., Giardina, B., Gill, S. J., and Brunori, M. (1982) Thermodynamics of oxygen binding to trout haemoglobin I and its oxidation intermediates. *J. Mol. Biol.* **160**, 531–543.
112. Wyman, J. Jr., Gill, S. J., Noll, L., Giardina, B., Colosimo, A., and Brunori, M. (1977) The balance sheet of a haemoglobin: thermodynamics of CO binding by haemoglobin trout I. *J. Mol. Biol.* **109**, 195–205.
113. Brunori, M., Giardina, B., Colosimo, A., Falcioni, G., and Gill, S. J. (1980) Temperature perturbation of the allosteric equilibrium in trout hemoglobin. *J. Biol. Chem.* **255**, 3841–3843.
114. Hofrichter, J., Henry, E. R., Szabo, A., Murray, L. P., Ansari, A., Jones, C. M., Coletta, M., Falcioni, G., Brunori, M., and Eaton, W. A. (1991) Dynamics of the quaternary conformational change in trout hemoglobin. *Biochemistry* **30**, 6583–6598.
115. Eaton, W. A., Henry, E. R., and Hofricther, J. (1991) Applications of linear free energy relations to protein conformational changes: the quaternary structural change of haemoglobin. *Proc. Natl. Acad. Sci. USA* **88**, 4472–4475.

116. Brunori, M., Coletta, M., Giardina, B., and Wyman, J. Jr. (1978) A macromolecular transducer as illustrated by trout hemoglobin IV. *Proc. Natl. Acad. Sci. USA* **75**, 4310–4312.
117. Wyman, J. Jr., Gill, S. J., Gaud, H. T., Colosimo, A., Giardina, B., Kuiper, H. A., and Brunori, M. (1978) Thermodynamics of ligand binding and allosteric transition in hemoglobins: reaction of Hb trout IV with CO. *J. Mol. Biol.* **124**, 161–175.
118. Giardina, B., Ascoli, F., and Brunori, M. (1975) Spectral changes and allosteric transition in trout haemoglobin. *Nature* **256**, 761–762.
119. Giardina, B., Giacometti, G. M., Coletta, M., Brunori, M., Giacometti, G., and Rigatti, G. (1978) A comparative approach to protein- and ligand-dependence of the Root effect for fish haemoglobins. *Biochem. J.* **175**, 407–412.
120. Coletta, M., Ascenzi, P., D'Avino, R., and di Prisco, G. (1996b) Proton-linked subunit kinetic heterogeneity for carbon monoxide binding to hemoglobin from *Chelidonichtys kumu*. *J. Biol. Chem.* **271**, 29859–29864.
121. Ball, E. G., Strittmatter, C. F., and Cooper, O. (1955) Metabolic studies on the gas gland of the swim bladder. *Biol. Bull.* **108**, 1–17.
122. Perutz, M. F., and Brunori, M. (1982) Stereochemistry of cooperative effects in fish and amphibian haemoglobins. *Nature* **299**, 421–426.
123. Nagai, K., Perutz, M. F., and Poyart, C. (1985) Oxygen binding properties of human mutant hemoglobins synthesized in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **82**, 7252–7255.
124. Bellelli, A., Brunori, M., Miele, A. E., Panetta, G., and Vallone, B. (2006) The allosteric properties of hemoglobin: insights from natural and site directed mutants. *Curr. Protein Pept. Sci.* **7**, 17–45.
125. Perutz, M. F., and Lehmann, H. (1968) Molecular pathology of human haemoglobin. *Nature* **219**, 902–909.
126. Morimoto, H., Lehmann, H., and Perutz, M. F. (1971) Molecular pathology of human haemoglobin: stereochemical interpretation of abnormal oxygen affinities. *Nature* **232**, 408–413.
127. Nagai, K., Luisi, B., Shih, D., Miyazaki, G., Imai, K., Poyart, C., De Young, A., Kwiatkowsky, L., Noble, R. W., Lin, S.-H., and Yu, N.-T. (1987) Distal residues in the oxygen binding site of haemoglobin studied by protein engineering. *Nature* **329**, 858–860.
128. Baldwin, J., and Chothia, C. (1979) Haemoglobin: the structural changes related to ligand binding and its allosteric mechanism. *J. Mol. Biol.* **129**, 175–220.
129. Vallone, B., Vecchini, P., Cavalli, V., and Brunori, M. (1993) Site-directed mutagenesis in hemoglobin: effect of some mutations at protein interfaces. *FEBS Lett.* **324**, 117–122.
130. Vallone, B., Bellelli, A., Miele, A. E., Brunori, M., and Fermi, G. (1996) Probing the $\alpha_1\beta_2$ interface of human hemoglobin by mutagenesis. Role of the FG-C contact regions. *J. Biol. Chem.* **271**, 12472–12480.
131. Miele, A. E., Santanchè, S., Travaglini-Allocatelli, C., Vallone, B., Brunori, M., and Bellelli, A. (1999) Modulation of ligand binding in engineered human haemoglobin distal pocket. *J. Mol. Biol.* **290**, 515–524.
132. Miele, A. E., Draghi, F., Arcovito, A., Bellelli, A., Brunori, M., Travaglini-Allocatelli, C., and Vallone, B. (2001) Control of heme reactivity by diffusion: structural basis and functional characterization in hemoglobin mutants. *Biochemistry* **40**, 14449–14458.
133. Perutz, M. F., Wilkinson, A. J., Paoli, M., and Dodson, G. G. (1998) The stereochemical mechanism of the cooperative effects in haemoglobin revisited. *Annu. Rev. Biophys. Biomol. Struct.* **27**, 1–34.
134. Brunori, M., Giardina, B., and Kuiper, H. A. (1982) Oxygen transport proteins. In *Inorganic Biochemistry* (Hill, H. A. O., ed.) pp. 126–182, vol. III, Royal Society of Chemistry, London.
135. Brunori, M., Coletta, M., and Giardina, B. (1985) Oxygen carrier proteins. In *Metalloproteins: Part 2, Metal Proteins with Non-redox Roles* (Harrison, P. M., ed.). pp. 263–331, McMillan, London.
136. Colosimo, A., Brunori, M., and Wyman, J. Jr. (1974) Concerted changes in an allosteric macromolecule. *Biophys. Chem.* **2**, 338–344.
137. Robert, C. H., Decker, H., Richey, B., Gill, S. J., Wyman, J. Jr. (1987) Nesting: hierarchies of allosteric interactions. *Proc. Natl. Acad. Sci. USA* **84**, 1891–1895.
138. Brunori, M., Giardina, B., and Bannister, J. V. (1979) Oxygen transport proteins. In *Inorganic Biochemistry* (Hill, H. A. O., ed.), pp. 159–209, vol. I, Royal Society of Chemistry, London.
139. Zolla, L., Coletta, M., Di Cera, E., Giardina, B., Kuiper, H., and Brunori, M. (1986) Discrimination of tertiary and quaternary Bohr effect in the oxygen binding of *Helix pomatia* β-hemocyanin. *Biophys. Chem.* **24**, 319–325.
140. Brunori, M. (1971) Kinetics of the reaction of *Octopus vulgaris* hemocyanin with oxygen. *J. Mol. Biol.* **55**, 39–48.
141. Kuiper, H. A., Antonini, E., and Brunori, M. (1977) Kinetic control of cooperativity in the oxygen binding of *Panulirus interruptus* hemocyanin. *J. Mol. Biol.* **116**, 569–576.
142. Kuiper, H. A., Brunori, M., and Antonini, E. (1978) Kinetics of the Bohr effect in the reaction of *Helix pomatia* β-hemocyanin with oxygen. *Biochem. Biophys. Res. Commun.* **82**, 1062–1069.
143. Kuiper, H. A., Coletta, M., Zolla, L., Chiancone, E., and Brunori, M. (1980) Root effect of *Panulirus interruptus* hemocyanin. *Biochim. Biophys. Acta* **626**, 412–416.
144. Antonini, E., Brunori, M., Colosimo, A., Kuiper, H. A., and Zolla, L. (1983) Kinetic and thermodynamic parameters for oxygen binding to the allosteric states of *Panulirus interruptus* hemocyanin. *Biophys. Chem.* **18**, 117–124.
145. Brunori, M. (1969) Kinetics of oxygen binding by octopus haemocyanin. *J. Mol. Biol.* **46**, 213–215.
146. Kuiper, H. A., Forlani, L., Chiancone, E., Antonini, E., Brunori, M., and Wyman, J. (1979) Multiple linkage in *Panulirus interruptus* hemocyanin. *Biochemistry* **18**, 5849–5854.
147. Zolla, L., and Brunori, M. (1983) A spectrophotometric method to determine the amount of CO bound to hemocyanin. *Anal. Biochem.* **133**, 465–469.
148. Giacometti, G. M., Focesi, A. Jr., Brunori, M., and Wyman, J. Jr. (1975) Effect of light on carbon monoxide binding by erythrocruorin. *J. Mol. Biol.* **98**, 333–339.
149. Giacometti, G. M., Focesi, A. Jr., Giardina, B., Brunori, M., and Wyman, J. Jr. (1975) Kinetics of binding of carbon monoxide to lumbricus erythrocruorin: a possible model. *Proc. Natl. Acad. Sci. USA* **72**, 4313–4316.
150. Brunori, M., Zolla, L., Kuiper, H. A., and Finazzi-Agrò, A. (1981) Reaction of carbon monoxide with hemocyanin: stereochemical effects of a non-bridging ligand. *J. Mol. Biol.* **153**, 1111–1123.
151. Zolla, L., Brunori, M., Richey, B., and Gill, S. J. (1985) Heterogeneous binding of oxygen and carbon monoxide to dissociated molluscan hemocyanin. *Biophys. Chem.* **22**, 271–280.
152. Kuiper, H. A., Zolla, L., Finazzi-Agrò, A., and Brunori, M. (1981) Interaction of lanthanide ions with *Panulirus interruptus* hemocyanin: evidence for vicinity of some of the cation binding sites. *J. Mol. Biol.* **149**, 805–812.
153. Antonini, E., Rossi-Fanelli, A., and Caputo, A. (1962) Studies on chlorocruorins. I. The oxygen equilibrium of *Spirographis* chlorocruorin. *Arch. Biochem. Biophys.* **97**, 336–342.
154. Qabar, A. N., Stern, M. S., Watz, D. A., Chin, J. T., Timkovich, R., Wall, J. S., Kapp, O. H., and Vinogradov, S. N. (1991) Hierarchy of globin complexes. The quaternary structure of the extracellular chlorocruorin of *Eudistylia vancouverii*. *J. Mol. Biol.* **222**, 1109–1129.
155. Gibson, Q. H., Bellelli, A., Regan, R., Sharma, P. K., and Vinogradov, S. N. (1992) Ligand binding by the chlorocruorin from *Eudistylia vancouverii*. *J. Biol. Chem.* **267**, 11977–11981.

156. Bellelli, A., Lendaro, E., Ippoliti, R., Regan, R., Gibson, Q. H., and Brunori, M. (1993) Ligand binding and slow structural changes in chlorocruorin from *Spirographis spallanzanii*. *Biochemistry* **32**, 7635–7643.
157. Bellelli, A., Giardina, B., Corda, M., Pellegrini, M. G., Cau, A., Condò, S. G., and Brunori, M. (1988) Sexual and seasonal variability of lobster hemocyanin. *Comp. Biochem. Physiol.* **91A**, 445–449.
158. Brunori, M., Kuiper, H. A., and Zolla, L. (1982) Ligand binding and stereochemical effects in hemocyanins. *EMBO J.* **1**, 329–331.
159. Benedetti, P. A., Bianchini, G., and Chiti, G. (1976) Fast scanning microspectrometry: an electrodynamic moving condenser method. *Appl. Optics* **15**, 2554.
160. Antonini, E., Brunori, M., Giardina, B., Benedetti, P. A., Grassi, S., and Gualtieri, P. (1982) Kinetics of reactions within single erythrocytes: studies by microspectrophotometry. *Haematologia* **15**, 3–15.
161. Brunori, M., Giardina, B., Antonini, E., Benedetti, P. A., and Bianchini, G. (1974) Distribution of the hemoglobin components of trout blood among the erythrocytes: observations by single cell spectroscopy. *J. Mol. Biol.* **86**, 165–169.
162. Antonini, E., Brunori, M., Giardina, B., Benedetti, P. A., Bianchini, G., and Grassi, S. (1978) Kinetics of the reaction with CO of human erythrocytes. Observations by single cell spectroscopy. *FEBS Lett.* **86**, 209–212.
163. Coletta, M., Giardina, B., Amiconi, G., Gualtieri, P., Benedetti, P. A., and Brunori, M. (1985) Kinetics of the reaction of intraerythrocytic hemoglobin by single cell microspectroscopy: effect of shape and osmolarity. *FEBS Letters* **190**, 217–220.
164. Brunori, M., Coletta, M., Bellelli, A., Evangelista, V., Benedetti, P. A., and Brumen, M. (1989) Microspectroscopy of red blood cells. *Haematologia* **22**, 69–78.
165. Coin, J. T., and Olson, J. S. (1979) The rate of oxygen uptake by human red blood cells. *J. Biol. Chem.* **254**, 1178–1190.
166. Wittenberg, J. B. (1966) The molecular mechanism of haemoglobin facilitated oxygen diffusion. *J. Biol. Chem.* **241**, 104–114.
167. Antonini, E., Brunori, M., Giardina, B., Benedetti, P. A., Bianchini, G., and Grassi, S. (1978) Single cell observations of gas reactions and shape changes in normal and sickling erythrocytes. *Biophys. J.* **24**, 187–195.
168. Coletta, M., Hofrichter, J., Ferrone, F. A., and Eaton, W. A. (1982) Kinetics of sickle haemoglobin polymerization in single red cells. *Nature* **300**, 194–197.
169. Coletta, M., Alayash, A. I., Wilson, M. T., Benedetti, P. A., Evangelista, V., and Brunori, M. (1988) Single cell microspectroscopy reveals that erythrocytes containing haemoglobin S retain a 'memory' of previous sickling cycles. *FEBS Lett.* **236**, 127–131.
170. Takano, T. (1984) Refinement of myoglobin and cytochrome *c*. In *Methods and Applications in Crystallographic Computing* (Hall, S. R., and Ashida, T., eds) pp. 262–272, Oxford University Press, Oxford.
171. Bolognesi, M., Rosano, C., Losso, R., Borassi, A., Rizzi, M., Wittenberg, J. B., Boffi, A., and Ascenzi, P. (1999) Cyanide binding to *Lucina pectinata* hemoglobin I and to sperm whale myoglobin: an X-ray crystallographic study. *Biophys. J.* **77**, 1093–1099.
172. Richard, V., Dodson, G. G., and Mauguen, Y. (1983) Human deoxyhaemoglobin-2,3-diphosphoglycerate complex low-salt structure at 2.5 Å resolution. *J. Mol. Biol.* **233**, 270–274.
173. Tame, J. R., Wilson, J. C., and Weber, R. E. (1996) The crystal structures of trout Hb I in the deoxy and carbonmonoxy forms. *J. Mol. Biol.* **259**, 749–760.
174. Holmes, M. A., Le Trong, I., Turley, S., Sieker, L. C., and Stenkamp, R. E. (1991) Structures of deoxy and oxy hemerythrin at 2.0 Å resolution. *J. Mol. Biol.* **218**, 583–593.
175. Magnus, K. A., Hazes, B., Ton-That, H., Bonaventura, C., Bonaventura, J., and Hol, W. G. (1994) Crystallographic analysis of oxygenated and deoxygenated states of arthropod hemocyanin shows unusual differences. *Proteins* **19**, 302–309.