Volume 307, number 1, 14–19 FEBS 11318 July 1992

Minireview

Protein dynamics

An overview on flash-photolysis over broad temperature ranges

Ernesto E. Di Iorio

Laboratorium für Biochemie I, Eidgenössische Technische Hochschule, Universitätsstrasse 16, 8092 Zurich, Switzerland

Received 9 June 1992

Ligand binding kinetics to heme-proteins between 40 and 300 K point to a regulatory role of protein dynamics. A protein-specific susceptibility of the heme-iron reactivity to dynamic fluctuations emerges from the distribution of reaction enthalpies derived from flash-photolysis measurements below ca. 180 K; we quantify it in terms of 'intramolecular viscosity', postulating that narrow low-temperature enthalpy distributions correspond to low internal viscosity and vice versa. The thermal evolution of ligand binding kinetics suggests, with other results, an interplay between high-frequency transitions of the amino acid side chains and low-frequency collective motions as a possible regulatory mechanism of protein dynamics.

Protein-dynamics; Heme-protein; Kinetics; Flash-photolysis; Protein-relaxation; Low-temperature

1. INTRODUCTION

The active site of a protein is normally well buried inside the polypeptide folding so that no free access is available for ligands or substrates to exchange between the solvent and the interior of the molecule [1,2]. Thanks to structural fluctuations, 'dynamic channels' are formed between the active site and the external medium and therefore function is guaranteed. In addition to this essential role, dynamic fluctuations appear to have also the more specific function of modulating the reactivity of proteins [3] and for this reason a steadily increasing number of scientists is attracted into this relatively new research field.

A great deal of the experimental work done to date on the correlation between protein dynamics and function rests on flash-photolysis measurements of the reaction between heme-proteins and small ligands (e.g. CO or O_2) over broad temperature ranges. The need for exploring temperatures that extend from physiological conditions down to few K is related to the fact that the energies involved in dynamic fluctuations of proteins are generally small, well below k_BT at room temperature.

Correspondence address: E.E. Di Iorio, Laboratorium für Biochemie I, Eidgenössische Technische Hochschule, Universitätsstrasse 16, 8092 Zurich, Switzerland

A detailed understanding of the link between dynamics and function requires also a deep knowledge of the three-dimensional organisation and of the structural fluctuations that the protein undergoes at equilibrium and during ligand binding. In this respect, the results of X-ray crystallography and NMR investigations, combined with molecular dynamics computer simulations, are of paramount importance [3,4]. A great contribution to our understanding of protein dynamics is provided also by light absorption, resonance Raman, and Mössbauer spectroscopy investigations [5–11].

In this short review most of the attention will be dedicated to experimental information obtained by flash-photolysis measurements on carbon monoxide binding to various proteins, studied at temperatures between 40 and 300 K. The essential features of the models presently available to analyse kinetic measurements over such broad temperature ranges will also be described.

2. KINETIC BEHAVIOUR OF PROTEINS AT CRYOGENIC TEMPERATURES

Since the pioneering work of the Urbana group on carbon monoxide binding to sperm whale (*Physeter catodon*) Mb at cryogenic temperatures [12], it is well established that the kinetic behaviour of proteins, under these thermal conditions, is characterised by non-exponential time courses. This feature, later found to apply to all heme-proteins investigated [13–17], has been explained in terms of sub-conformational heterogeneity

 Published by Elsevier Science Publishers B.V.

arising from the fact that, at cryogenic temperatures, structural fluctuations are no longer possible and therefore each protein molecule is unique in terms of its three dimensional organisation and, in turn, of its ligand-binding kinetics [12,18]. Another implication of the absence at cryogenic temperatures of structural fluctuations is that, after photolysis, the ligand remains trapped in the vicinity of the active site and rebinding can only occur from within the heme pocket [12,18]. Based on this overall picture, in a flash-photolysis measurement the fraction $N(t)$ of hemes that, at time t after photolysis, has not yet recombined with the ligand can be described by

$$N(t) = \int_0^\infty dk \cdot p(k) \cdot \exp[-k \cdot t] \quad (1)$$

where $p(k)dk$ represents the probability of finding a protein molecule in a state characterised by a reaction rate between k and $k+dk$. For the analysis of experimental data the temperature dependence of k is assumed to follow Arrhenius' law

$$k = k_0 \cdot \exp[-H/RT] \quad (2)$$

and the $p(k)$ in Eq. **(1)** is considered, for simplicity, to arise only from a distribution of enthalpy barriers, while the pre-exponential k_0 is assumed to have a discrete value. Under these conditions $N(t)$ becomes

$$N(t) = \int_0^\infty dH \cdot g(H) \cdot \exp[-k_0 \cdot \exp(-\frac{H}{RT}) \cdot t] \quad (3)$$

and the probability distribution can in principle be derived, by numeric inversion of the integral, from the experimental values of $N(t)$, t, and T. The numeric inversion of Eq. **(3)** tends to be a highly unstable procedure and therefore a parameterization of the $g(H)$ distribution becomes necessary. For this purpose several approaches have been proposed [16,19,20] allowing Eq. **(3)** to be successfully used for the analysis of flash-photolysis data [12–21].

3. THE LINK BETWEEN CRYOGENIC AND PHYSIOLOGICAL TEMPERATURES

A correlation between the distribution of ligand binding enthalpies $g(H)$, obtained from flash-photolysis measurements at cryogenic temperatures, and the dynamic fluctuations of proteins under physiological conditions is implicity assumed in the above interpretation of the non-exponential time-courses for low-temperature kinetics. Qualitatively speaking this correlation is confirmed by the finding that proteins with distinct structural and functional properties also display distinct enthalpy distributions for low-temperature CO binding. Thus, myoglobins are generally characterised by broad distributions that peak at enthalpies of at least 10 kJ/mol, while hemoglobin chains have narrower $g(H)$

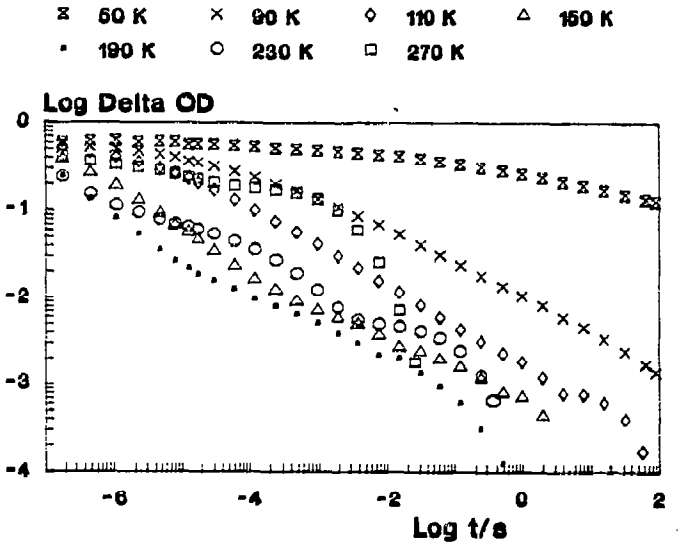

Fig. 1. Thermal evolution of the CO-binding kinetics to phosphate-free Carp Hb in 50 mM Cl-Bis-Tris pH 8.2, containing 75% glycerol (De Iorio, E.E., Yu, W. and Noble, R.W., unpublished results).

peaked at around 5 kJ/mol [13,16,22]. Based also on results obtained from light absorption spectroscopy measurements as function of temperature, it has been postulated that the peak position of the $g(H)$ is primarily determined by the packing of the protein matrix around the active site, while its width is mainly determined by the 'flexibility' or 'internal viscosity' of the macromolecule [16]. The low-temperature light-absorption properties and the CO-binding kinetics of the hemoglobin subunits show that the α chains have a higher 'flexibility' than the β [5,16] and, in view of this difference, it has been proposed that cooperativity might result from the presence, within a protein, of domains characterised by different 'intrinsic viscosity' [16,22]. The difference between the hemoglobin subunits in terms of internal viscosity, as judged from low-temperature $g(H)$, is further amplified by quaternary conformational changes of the tetramer. The α chains are a little affected by the R→T transition, while the β subunits respond to this quaternary conformational change with a broadening and a shift to higher enthalpies of the low-temperature $g(H)$ (Di Iorio, E.E., Yu, W., Calonder, C., Tavernelli, I., Winterhalter, K.H., 1992, manuscript in preparation).

The above experimental evidence is sufficient to state the existence of a correlation between dynamic properties and low-temperature $g(H)$ distribution for ligand binding. However, this link does not provide any quantative mean of deriving the dynamic behaviour of a protein, under physiological conditions, from its kinetic properties at cryogenic temperatures. Valuable information in this respect can be obtained from an analysis of the thermal evolution of the ligand-binding kinetics

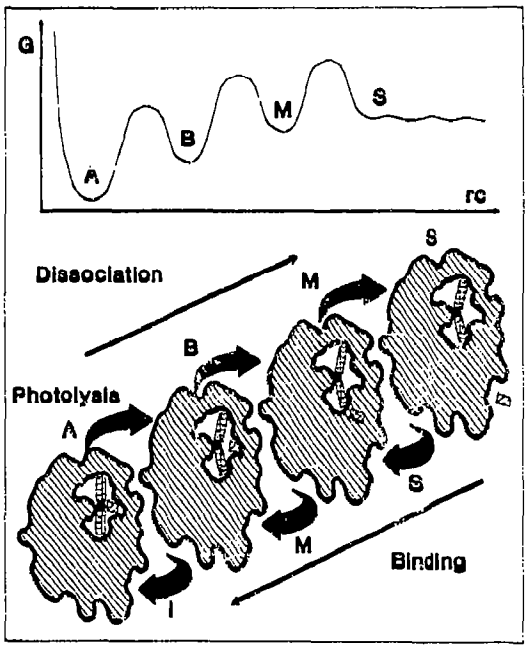

Fig. 2. Schematic representation of the sequential multi-barrier model. Three processes are postulated for the ligand-binding reaction and are indicated with the letters I, (internal), M (matrix), and S (solvent).

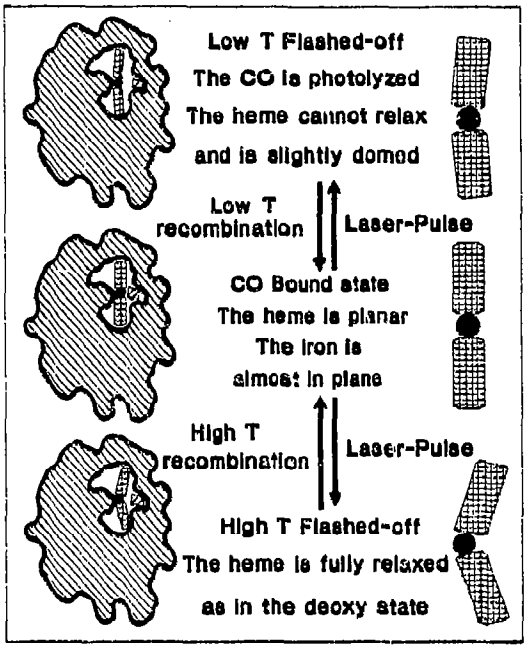

Fig. 3. The relaxation model as proposed by Steinbach et al. [25]. At low temperatures the 'frozen' protein inhibits a complete relaxation of the heme. This results in a lower activation barrier between the dissociated and the bound states, compared to when the heme is fully relaxed (high T).

from cryogenic to room temperatures. A striking feature that emerges from this analysis is that, below ca. 180 K, the ligand-binding reaction speeds up as the temperature rises, according to Arrhenius' law, but above this 'critical point' there is an inversion of the trend and the overall reaction velocity decreases with increasing temperature [18,23–25]. An example of this behaviour is given in Fig. 1, where CO-binding time courses to carp Hb in the R state, at few representative temperatures, are illustrated. Another peculiarity of the thermal evolution of the ligand-binding kinetics is that, while at cryogenic temperatures the traces, even though non-exponential, are smooth, at higher temperatures they reveal the presence of more than one process. In the example of Fig. 1, already at 190 K two processes can be easily identified and, starting from 230 K a third one, exponential in time, sets in. The behaviour below ca. 180 K is consistent with the idea that ligand binding after photolysis occurs in a 'frozen protein' and only within the heme-pocket, as described above. To explain the experimental results at higher temperatures two major lines of thoughts have been followed, one based on a sequential multibarrier scheme [18,24] and the other on the so-called relaxation model [25,26].

3.1. *The sequential multibarrier model*

The appearance at ca. 180 K of a second kinetic process, in addition to that observed at cryogenic temperatures (Fig. 1), is assumed to be associated with the diffusion of the ligand through the protein matrix and considered to be responsible for the progressive decrease of the overall ligand-binding velocity. The new kinetic process is thought to arise from the penetration of the ligand from the heme pocket into the macromolecular matrix, due to increased thermal fluctuations within the protein. A further temperature rise will cause an additional increase of the internal mobility of the protein and ligand molecules will start exchanging between the active site and the solvent, as shown by the appearance of a third process. The sequential multibarrier model, schematically illustrated in Fig. 2, describes the ligand-binding reaction in terms of one bound state (A), and three dissociated states in which the ligand can either be in the heme pocket (B), in the protein matrix (M), or in the solvent (S). Within this model, the extrapolation of the kinetic behaviour from cryogenic to physiological temperatures is based on the assumption that ligand binding is regulated by the innermost kinetic step (B→A) and that the rate coefficient for the physiologically relevant reaction is directly proportional to k_{BA}, the average rate constant for the B→A process determined from the low-temperature $g(H)$ [23,24].

The multibarrier model has the advantage of giving a plausible explanation for the presence, at high temperatures, of three kinetic processes. However, the assumption that the distribution of activation enthalpies is practically temperature independent is certainly an oversimplification; in the light of our present knowl-

edge, it is hard to accept that the onset of molecular fluctuations at around 180 K has the only effect of allowing the migration of ligand molecules through the protein matrix, without affecting the energy of the ligand-binding process at the heme.

3.2. *The relaxation model*

Another approach to describe the thermal evolution of ligand-binding kinetics is to relate the inverse proportionality between temperature and reaction velocity, observed starting around 180 K, not to the onset of the matrix process, but to an increase of the free energy of the reaction as a consequence of larger structural fluctuations of the macromolecule. Agmon and Hopfield [26] introduced first this concept and Steinbach at al. [25] have re-elaborated it more recently. The envisaged scenario, schematically illustrated in Fig. 3, is that at cryogenic temperatures ligand photolysis is accompanied only by a partial displacement of the iron from the heme plane, compared to the equilibrium-deoxy situation. The different degree of heme-relaxation upon ligand dissociation is thought to be characterised by different ligand-binding free energies and therefore, at cryogenic temperatures, the $g(H)$ distribution is expected to peak at smaller enthalpies compared to high temperatures. To extrapolate from the low-temperature $g(H)$ the ligand-binding kinetics above the critical temperature of ca. 180 K, Steinbach et al. [25] have made the following basic assumptions: (i) the $g(H)$ changes its peak position as function of temperature, but its shape is temperature independent; (ii) the ΔH between the 'frozen' and the fully 'relaxed' states is proportional to the degree of iron displacement from the heme-plane; this can be determined from the shift, between the low-temperature photolysed and the equilibrium-deoxy states, of the peak position of the near-IR light absorption band, present in the deoxy spectrum of heme proteins at around 760 nm; (iii) the relaxation from the low- to the high-temperature heme-doming is non-exponential in time and does not follow an Arrhenius' temperature dependence. An important feature of this model is that, above ca. 180 K, the velocity of ligand binding is not only temperature, but also time dependent [25].

Even though considering the shape of the $g(H)$ to be unaffected by temperature is certainly an oversimplification, the approach followed in the relaxation model is more realistic than that used in the sequential multi-barrier scheme and allows a quantitative description of the CO-binding kinetics to sperm whale Mb [25]. A draw-back of the relaxation model, in its present form, is that it cannot explain the presence of three kinetic processes above ca. 220 K (Fig. 1) since only two sequential energy barriers are assumed, one discrete corresponding to the penetration of the ligand from the solvent into the protein (S→B) and the other, enthalpy distributed, for the actual ligand binding to the heme-iron (B→A).

4. SOME PERSPECTIVES

One important goal in biochemistry and molecular biology is the understanding of the structure–function relationships in proteins. Based on our present knowledge, the three-dimensional folding of a polypeptide is not sufficient to decide how the functional properties of the active site are determined and regulated; it is not even clear why proteins are so large. Answers to these basic questions will possibly be available once a description of biochemical processes, based on the interplay between structure, dynamics and function, will be possible. Computer simulations of protein dynamics will play an increasingly important role, but the results of simulations will have to be tested at the experimental level. Flash photolysis will remain an essential tool in this respect, since it allows the analysis of proteins over broad temperature ranges and while they are 'functioning' and not just in a 'resting state'. The possibility of extracting detailed information about the dynamics of structural fluctuations from flash photolysis measurements is therefore of paramount importance and the heme-relaxation model proposed by Steinbach et al. [25] represents a good starting point for the development of more general approaches. The available experimental information can help in defining the direction in which to move along this main line of thought.

In remarkable agreement with the results on CO-binding kinetics, Mössbauer spectroscopy measurements on sperm whale Mb [11] show that the thermal evolution of the mean sqare displacement $\langle x^2 \rangle$ of the heme-iron follows Debye's law up to about 180 K, just like an ordinary solid, but that its susceptibility to temperature drastically increases above this point. Also a study on the temperature dependence of the Soret light absorption band of sperm whale Mb, adult human Hb, and its α and β chains demonstrates that the coupling of the porphyrin $\pi \rightarrow \pi^*$ electronic transition with high-frequency modes of the tetrapyrrole, detected by resonance Raman spectroscopy, and with low-frequency modes that involve the entire protein, is characterised by strong anharmonic contributions above ca. 180 K [5]. As clearly stated by the authors of this investigation, "anharmonicity of nuclear motions is an obvious prerequisite for jumping among conformational substates of the protein". Deviations above ca. 180 K from the harmonic thermal behaviour of atomic fluctuations in proteins have also been shown by inelastic neutron scattering measurements [27] and by computer simulations [28,29]. The picture that emerges from all these results is that in proteins a new set of intramolecular motions start to be populated around 180 K.

The averaged mean square displacements of the backbone atoms and of the amino acid side chains in sperm whale Mb become temperature independent below ca. 80 K, while depending on temperature in an aproximately linear way above this value [11]. A discontinuity

at around 80 K has also been observed in the thermal evolution of the CO-binding kinetics to a number of heme-proteins [16] (Di Iorio, unpublished results).

A plausible explanation for these observations is that 80 K and 180 K represent respectively critical temperatures for rotational transitions of amino acid side chains and for low-frequency collective motions. An interdependence of the two sets of motions, probably mediated by secondary interactions, is very likely and would rationalise the hierarchical organisation of structural fluctuations proposed by Ansari et al. [30]. On the other hand, *X*-ray diffraction analysis of sperm whale Mb, carried between 40 and 300 K, show that structural fluctuations involve diffusion-like collecitve motions which are completely abolished upon freezing of the hydration water or drying of the sample [11]. The same conclusion is also reached by molecular dynamics simulations [29].

In the light of this interpretation, it appears that attributing the increase in the ligand-binding enthalpy above ca. 180 K to the relaxation of the heme, as proposed by Steinbach et al. [25], represents a limitation to the general applicability of the results obtained from protein dynamics investigations on heme proteins. The concept of 'internal viscosity', introduced above to describe the difference in the dynamic properties between the myoglobins and the hemoglobins subunits, combined with the scenario just outlined, provide the starting conditions for the development of a new relaxation model. A recent investigation by Ansari et al. [31] on the kinetics of CO-binding to sperm whale Mb as a function of solvent viscosity represents a very promising step in this direction.

The interplay between high-frequency local modes and low-frequency collective motions, mediated by secondary interactions, appears to be an efficient mean for the control of protein reactivity through dynamic fluctuations. Relatively high energies can be dislocated over large areas of the molecule and therefore regulation can be achieved without interfering with protein stability. This could also explain why proteins are so large. In keeping with this view, a recent study on the dynamic properties of the horse myoglobin fragment including residues 32 to 139 (ca. 2/3 of the native molecule) shows that the structural oscillations occurring in this 'mini-Mb' upon ligand binding and dissociation are too large for the system to be energetically and functionally efficient [32].

Acknowledgements: I am very thankful to Yu Weiming for many stimulating discussions and to the groups of Lorenzo Cordone and William A. Eaton for providing, prior to publication, the manuscripts by Di Pace et al. (1992) and by Ansari et al. (1992).

Support by the Swiss National Science Foundation Grant 31.9432.88 and by the ETH special credit 03586/41-1080.5 is also acknowledged.

REFERENCES

[1] Perutz, M.F. and Matthews, F.S. (1966) J. Mol. Biol. 21, 199–201.
[2] Case, D.A. and Karplus, M. (1979) J. Mol. Biol. 132, 343–368.
[3] Karplus, M. and Petsko, G.A. (1990) Nature 347, 631–639.
[4] Wüthrich, K. (1986) In: NMR of Proteins and Nucleic Acids, Wiley, New York.
[5] Di Pace, A., Cupane, A., Leone, M., Vitrano, E. and Cordone, L. (1992) Biophys. J. (in press).
[6] Ormos, P., Ansari, A., Braunstein, D., Cowen, B.R., Frauenfelder, H., Hong, M.K., Iben, I.E.T., Sauke, T.B., Steinbach, P.J. and Young, R.D. (1990) Biophys. J. 57, 191–199.
[7] Srajer, V., Schomacker, K.T. and Champion, P.M. (1986) Phys. Rev. Lett. 57, 1267–1270.
[8] Spiro, T.G., Smulevich, G., Su, C. (1990) Biochemistry 29, 4497–4508.
[9] Winkler, H., Franke, M., Trautwein, A.X. and Parak, F. (1990) Hyperfine Interactions 58, 2405–2412.
[10] Krupyanskii, Y.F., Goldanskii, V.I., Nienhaus, G.U. and Parak, F. (1990) Hyperfine Interactions 53, 59–74.
[11] Parak, F. (1989) NATO Advanced Science Institutes (ASI) Ser. A. 178, 197–221.
[12] Austin, R.H., Beeson, K., Eisenstein, L., Frauenfelder, H., Gunsalus, I.C. and Marshall, V.P. (1973) Science 181, 541–543.
[13] Alberding, N., Shirley, S.C., Eisenstein, L., Frauenfelder, H., Good, D., Gunsalus, I.C., Nordlund, T.M., Perutz, M.F., Reynolds, A.H. and Sorensen, L.B. (1978) Biochemistry 17, 43–51.
[14] Doster, W., Bowne, S.F., Frauenfelder, H., Reinisch, L. and Shyamsunder, E. (1987) J. Mol. Biol. 194, 299–312.
[15] Stetzkowski, F., Banerjee, R., Marden, M.C., Beece, D.K., Bowne, S.F., Doster, W., Eisenstein, L., Frauenfelder, H., Reinish, L., Shyamsunder, E. and Jung, C. (1985) J. Biol. Chem. 260, 8803–8809.
[16] De Iorio, E.E., Hiltpold, U.R., Filipovic, D., Winterhalter, K.H., Gratton, E., Vitrano, E., Cupane, A., Leone, M. and Cordone, L. (1991) Biophys. J. 59, 742–754.
[17] Richter, C. and Di Iorio, E.E. (1991) Frontiers in Biotransformation, 5 (K. Ruckpaul & H. Rein, Eds.) Akademie Verlag, Berlin, pp. 72–93.
[18] Austin, R.H., Beeson, K.W., Eisenstein, L., Frauenfelder, H. and Gunsalus, I.C. (1975) Biochemistry 14, 5355–5373.
[19] Young, R.D. and Bowne, S.F. (1984) J. Chem. Phys. 81, 3730–3737.
[20] Srajer, V., Reinish, L. and Champion, P.M. (1988) J. Am. Chem. Soc. 110, 6656–6670.
[21] Alberding, N., Austin, R.H., Shirley, S.C., Eisenstein, L., Frauenfelder, H., Gunsalus, I.C. and Nordlund, T.M. (1976) J. Chem. Phys. 65, 4701–4711.
[22] Winterhalter, K.H. and Di Iorio, E.E. (1987) Acta Haematol. 78, 90–94.
[23] Doster, W., Beece D., Bowne, S.F., De Iorio E.E., Eisenstein, L., Frauenfelder, H., Reinish, L., Shyamsunder, E., Winterhalter, K.H. and Yue, K.T. (1982) Biochemistry 21, 4832–4839.
[24] Ansari, A., De Iorio, E.E., Dlott, D.D., Frauenfelder, H., Iben, I.E.T., Langer, P., Roder, H., Sauke, T.B. and Shyamsunder, E. (1986) Biochemistry 25, 3139–3146.
[25] Steinbach, P.J., Ansari, A., Berendzen, J., Braunstein, D., Chu, K., Cowen, B.R., Ehrenstein, D., Frauenfelder, H., Johnson, J.B., Lamb, D.C., Luck, S., Mourant, J.R., Nienhaus, G.U., Ormos, P., Philipp, R., Scholl, R., Xie, A. and Young, R.D. (1991) Biochemistry 30, 3988–4001.
[26] Agmon, N. and Hopfield, J.J. (1983) J. Chem. Phys. 79, 2042–2053.
[27] Doster, W.S., Cusack, S. and Petry, W. (1989) Nature 337, 754–756.

[28] Loncharic, R.J. and Brooks, B.R. (1990) J. Mol. Biol. 215, 439–455.
[29] Smith, J., Kuczera, K. and Karplus, M. (1990) Proc. Natl. Acad. Sci. USA 87, 1601–1605.
[30] Ansari, A., Berendzen, J., Bowne, S.F., Frauenfelder, H., Iben, I.E.T., Sauke, T.E., Shyamsunder, E. and Young, R.D. (1985) Proc. Natl. Acad. Sci. USA 82, 5000–5002.
[31] Ansari, A., Jones, C.M., Henry, E.R., Hofrichter, J. and Eaton, W.A. (1992) Science (in press).
[32] Di Iorio, E.E., Yu, W., Calonder, C., Winterhalter, K.H., De Sanctis, G., Falcioni, G., Ascoli, F., Giardina, B. and Brunori, M. (1992) Proc. Natl. Acad. Sci. USA (submitted).