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[14] Protein Dynamics and Hydration

By HANS FRAUENFELDER and ENRICO GRATTON

Proteins are dynamic and not static systems; their internal motion is crucial for their function. Water is essential for protein motions and consequently also for protein function. Beyond these general statements, a full understanding of the relation between motion and structure and between hydration and motions is not yet available, and most of the essential features of the role of water for biological action remain to be elucidated. In this chapter we outline the general aspects of protein states and protein motions and discuss how water may be involved. This chapter is not a comprehensive guide to dynamics and hydration, but a modest road map to further work. To be specific, we will explain concepts and processes by using simple examples, but we believe that the phenomena are general and occur in various disguises in most proteins and probably also in nucleic acids.

Protein dynamics has been treated in a number of reviews.¹⁻¹⁵ We refer to these reviews for more extensive lists of references and details.

States and Motions in Proteins

A *working* protein can exist in more than one state. Consider as a simple example myoglobin (Mb). To perform its function, Mb binds and releases oxygen and the two states are deoxy- and oxymyoglobin (deoxyMb and MbO₂). The different states will have different properties and usually different conformations. Even a resting protein in a given state

¹ G. Careri, P. Fasella, and E. Gratton, *Crit. Rev. Biochem.* **3**, 141 (1975).

² G. Careri, P. Fasella, and E. Gratton, *Annu. Rev. Biophys. Bioeng.* **8**, 69 (1979).

³ F. R. N. Gurd and T. M. Rothgeb, *Adv. Protein Chem.* **33**, 73 (1979).

⁴ R. J. P. Williams, *Biol. Rev.* **54**, 389 (1979).

⁵ A. Cooper, *Sci. Prog. Oxford* **66**, 473 (1981).

⁶ M. Karplus and J. A. McCammon, *Crit. Rev. Biochem.* **9**, 293 (1981).

⁷ M. R. Eftink and C. A. Ghiron, *Anal. Biochem.* **114**, 199 (1981).

⁸ P. Debrunner and H. Frauenfelder, *Annu. Rev. Phys. Chem.* **33**, 283 (1982).

⁹ G. R. Welch, B. Somogyi, and S. Damjanovich, *Prog. Biophys. Mol. Biol.* **39**, 109 (1982).

¹⁰ C. Woodward, I. Simon, and E. Tuchsén, *Mol. Chem. Biochem.* **48**, 135 (1982).

¹¹ M. Karplus and J. A. McCammon, *Annu. Rev. Biochem.* **53**, 263 (1983).

¹² G. Wagner, *Q. Rev. Biophys.* **16**, 1 (1983).

¹³ S. W. Englander and N. R. Kallenbach, *Q. Rev. Biophys.* **16**, 521 (1984).

¹⁴ J. A. McCammon, *Rep. Prog. Phys.* **47**, 1 (1984).

¹⁵ G. A. Petsko and D. Ringe, *Annu. Rev. Biophys. Bioeng.* **13**, 331 (1984).

FIG. 1. Schematic representation of states, substates, equilibrium fluctuations (EF), and functionally important motions (FIMs).

will not remain in a unique conformation, but will fluctuate among a large number of conformational substates.¹⁶⁻¹⁹ Conformational substates have the same coarse overall structure, but differ in detail; all perform the same function, but possibly with different rates. States and substates are schematically shown in Fig. 1. The two states, MbO_2 and deoxyMb, differ clearly: in MbO_2 the ligand is bound to the heme iron, the heme is nearly planar, and the iron is in the heme plane and has spin 0. In deoxyMb, the iron has moved out of the heme plane and has spin 2, the heme is domed, and the overall protein structure has changed.^{19,20} The various substates are distinguished by small changes in the protein structure.^{19,21} A side chain may have rotated, some hydrogen bonds may have shifted, a single helix may be displaced, or the entire protein may be rearranged.

Two different types of motions can be distinguished in the simple case of Fig. 1, equilibrium fluctuations (EF) and functionally important motions (FIMs). In EF, a resting protein moves from one substate to another, but does not change its state. A FIM, in contrast, describes the motion from one state to the other. The magnitude, but not the time dependence, of the equilibrium fluctuations is determined by equilibrium thermodynamics.^{16,17} FIMs, on the other hand, are not characterized by equilibrium thermodynamics. At first sight, the rates of EF and of FIMs therefore appear to be unrelated. Fortunately, however, there exists a powerful connection, the fluctuation-dissipation theorem. The connec-

¹⁶ A. Cooper, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2740 (1976).

¹⁷ A. Cooper, *Prog. Biophys. Mol. Biol.* **44**, 181 (1984).

¹⁸ R. H. Austin, K. W. Beeson, L. Eisenstein, H. Frauenfelder, and I. C. Gunsalus, *Biochemistry* **14**, 5355 (1975).

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²⁰ S. E. V. Phillips, *J. Mol. Biol.* **142**, 531 (1980).

²¹ H. Hartmann, F. Parak, W. Steigemann, G. A. Petsko, D. Ringe, and H. Frauenfelder, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4967 (1982).

FIG. 2. Fluctuation-dissipation theorem. Left: Equilibrium fluctuations. The characteristic rate of EF can be obtained from the autocorrelation function of $L(t)$. Right: Approach to equilibrium from a state far from equilibrium. The characteristic rate of EF can also be obtained from the exponential time dependence of $L(t)$.

tion between an equilibrium and nonequilibrium property was introduced by Einstein in his paper on Brownian motion²² where he connected the diffusion coefficient D to the friction coefficient f through the relation $D = k_B T/f$. Here k_B is the Boltzmann constant and T the temperature.²³ The theorem was stated more generally by Callen and Welton²⁴ and later generalized by Kubo and others.²⁵⁻²⁸ Because the theorem is important in the treatment of protein dynamics, we will give a simple example.

Consider a molecule that can exist in either one of two states of equal energy, L and R . The two states are represented by the two wells shown in Fig. 2. In equilibrium, an ensemble of N molecules will on the average occupy both wells equally, $\langle L \rangle = \langle R \rangle = N/2$. The instantaneous values, $L(t)$ and $R(t)$, will, however, fluctuate around the mean value, as indicated in Fig. 2b, and these fluctuations will occur with a rate $2k$, as indicated in Fig. 2a. In the nonequilibrium case, all molecules will initially be in one well, say L (Fig. 2c). The ensemble will approach equilibrium exponentially in time, with a rate $2k$, as shown in Fig. 2d. (In Fig. 2d, we assume N to be so large that the fluctuations are not visible.) The example shows

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²³ R. S. Berry, S. A. Rice, and J. Ross, "Physical Chemistry." Wiley, New York, 1980.

²⁴ H. B. Callen and T. A. Welton, *Phys. Rev.* **83**, 34 (1951).

²⁵ R. Kubo, *Rep. Prog. Phys.* **29**, 255 (1966).

²⁶ M. Suzuki, *Prog. Theor. Phys.* **56**, 77 (1976).

²⁷ L. D. Landau and E. M. Lifshitz, "Statistical Physics." Pergamon, Oxford, 1959.

²⁸ M. Lax, *Rev. Mod. Phys.* **32**, 25 (1960).

that the rate of equilibrium fluctuations and the rate of the approach to the equilibrium from a state far from equilibrium are the same. The example given in Fig. 2 is particularly simple because only two states are involved and both have equal energies. The situation in a protein (Fig. 3) is more complex; many substates exist and they do not all have equal energies. States and substates may not all be connected by thermal fluctuations. Nevertheless, we expect that EF and FIMs are still related through generalized fluctuation-dissipation theorems. The relations provide the basis for the study of the characteristic time of spontaneous fluctuations using perturbation methods.

Large equilibrium fluctuations and FIMs are possible because of the unique construction of proteins. The covalently bonded backbone is held in the folded tertiary structure by relatively weak forces so that the flexibility is not severely restricted. The physical origin of the protein flexibility arises from the structure of the polypeptide chain which allows almost free rotation around the ϕ and ψ angles. Rotations of several tens of degrees can be obtained with energies on the order of a few kJ/mol. High barriers to rotations are provided by the π bonding of the peptide group and of the aromatic rings of some residues in tyrosine, tryptophan, histidine, and phenylalanine. The proline residue constitutes a special case, and this residue is important in determining the rigidity of the polypeptide

FIG. 3. Hierarchical protein model. One-dimensional representation of the Gibbs free energy as a function of a conformational coordinate for the three tiers, CS^1 , CS^2 , and CS^3 .

chain. The major obstacle to rotations arises from van der Waals repulsion resulting from the collision with other atoms of the protein structure. Hydrogen bonding between residues in α -helices and β -sheets and S-S covalent bonds of cysteine residues further constrain rotations.

Hierarchy of Substates and Motions

A detailed look at a particular protein, Mb, reveals that the scheme of Fig. 1 is too restricted and that substates and motions appear to possess a hierarchical structure.²⁹ The structure can be described in Fig. 3. A protein in a given state, say MbO₂, can exist in a large number of conformational substates (CS). The barriers between the various CS are of the order of 100 kJ/mol. Substates (CS¹) are represented at the top of Fig. 3 by drawing the Gibbs energy of the protein as a function of a conformational coordinate. A look at a given potential minimum with higher resolution indicates that it encompasses a very large number of local minima, the conformational sub-substates (CS²). These CS² are separated by barriers with energies ranging from 10 to 50 kJ/mol. Each CS² again shows a structure and is divided into sub-sub-substates (CS³). The barriers between these CS³ are of the order of a few kJ/mol or less.

The experimental evidence for various classes (tiers) of states comes from a variety of experiments. Many aspects remain to be explored and the following remarks should only convey some ideas of how substates and motions can be studied.²⁹ The existence of CS³, substates separated by very small energy barriers, follows from measurements of the specific heat and dielectric relaxation at temperatures as low as 0.2 K.³⁰⁻³² Evidence for the second-tier CS² comes, for instance, from Mössbauer experiments³³⁻³⁵ and from NMR.³⁶ Evidence for the substates of the first tier, CS¹, is provided by the nonexponential time dependence of the binding of

²⁹ H. Frauenfelder, in "Structure and Dynamics of Nucleic Acids, Proteins and Membranes" (E. Clementi, G. Corongiu, M. H. Sarma, and R. H. Sarma, eds.). Adenine, New York, 1985.

³⁰ G. P. Singh, H. J. Shink, H. V. Lohneysen, F. Parak, and S. Hunklinger, *Z. Phys. B* **55**, 23 (1984).

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³² L. Genzel, F. Kremer, A. Poglitsch, and G. Bechtold, *Biopolymers* **22**, 1715 (1983).

³³ F. Parak, E. N. Frolov, R. L. Mössbauer, and V. I. Goldanskii, *J. Mol. Biol.* **145**, 825 (1981).

³⁴ H. Keller and P. G. Debrunner, *Phys. Rev. Lett.* **45**, 66 (1980).

³⁵ E. R. Bauminger, S. G. Cohen, I. Nowik, S. Ofer, and J. Yariv, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 736 (1983).

³⁶ E. R. Andrew, D. J. Bryant, and E. M. Cashell, *Chem. Phys. Lett.* **69**, 551 (1980).

carbon monoxide and oxygen to heme proteins.^{18,37} Pressure titration experiments³⁸ demonstrate that transitions among the substates CS¹ do not occur below 210 K. Flash photolysis data on the β -chain of hemoglobin Zurich imply that binding of CO is not exponential in time below about 250 K.³⁹

The hierarchy of three tiers shown in Fig. 3 represents the present knowledge in myoglobin. It is possible that more tiers exist even in Mb, and it is not known at present if other proteins possess the same number of tiers. The assignment of the various tiers of substates to specific structural elements of the protein is also not yet unambiguous. It is likely that the lowest tier, CS³, is related to the motions of a few atoms or small groups; CS² may be connected to motions of larger units like helices, and CS¹ may involve the rearrangement of the entire protein molecule and the hydration layer. The existence of various tiers of substates may be linked to the existence of a hierarchy of domains within proteins.⁴⁰⁻⁴²

The hierarchy of substates shown in Fig. 3 leads to a hierarchy of motions, both in equilibrium and during a function. A protein with three tiers of substates will undergo three types of equilibrium fluctuations, EF1, EF2, and EF3. At very low temperatures, say below 50 K, only transitions among the substates CS³ of the lowest tier will occur. If these equilibrium fluctuations, denoted by EF3, were to proceed entirely by classical Arrhenius transitions, they would cease in the limit $T \rightarrow 0$. The observation that the specific heat of metmyoglobin approaches a $T^{1.3}$ dependence below 0.4 K implies that transitions still take place, even at these very low temperatures.³⁰ Quantum mechanical tunneling must be responsible for the EF3.^{31,43-47} As the temperature is increased above about 50 K, transitions among the substates CS² of the second tier will begin. In the simplest case, the EF3 will at these temperatures already be so fast that the different CS³ can no longer be distinguished. They are

³⁷ H. Frauenfelder, this series, Vol. 54, p. 28.

³⁸ L. Eisenstein and H. Frauenfelder, in "Frontiers of Biological Energetics," Vol. I, p. 680. Academic Press, New York, 1978.

³⁹ D. D. Dlott, H. Frauenfelder, P. Langer, H. Roder, and E. E. DiIorio, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6239 (1983).

⁴⁰ G. D. Rose, *J. Mol. Biol.* **134**, 447 (1979).

⁴¹ J. Janin and S. S. Wodak, *Prog. Biophys. Mol. Biol.* **42**, 21 (1983).

⁴² W. S. Bennett and R. Huber, *CRC Crit. Rev. Biochem.* **15**, 291 (1982).

⁴³ P. W. Anderson, B. I. Halperin, and L. M. Varma, *Philos. Mag.* **25**, 1 (1972).

⁴⁴ W. A. Phillips, *J. Low Temp. Phys.* **7**, 351 (1972).

⁴⁵ W. A. Phillips, *Top. Curr. Phys.* **24**, 1 (1981).

⁴⁶ D. DeVault, "Quantum Mechanical Tunnelling in Biological Systems," 2nd Ed. Cambridge Univ. Press, New York, 1984.

⁴⁷ V. I. Goldanskii, *Annu. Rev. Phys. Chem.* **27**, 85 (1976).

“blurred” and their properties can be averaged. Each substate CS^2 appears as a simple potential well. As the temperature is further increased, the EF2 will become faster and at about 200 K, each CS^1 will appear as a simple substate. At about 200 K, transitions among the CS^1 , the substates of the first tier, set in. At about 300 K, these EF1 will be so fast that the protein appears as a single state, corresponding to the dashed potential enveloping the CS^1 in Fig. 3.

The picture of equilibrium fluctuations as described so far requires some additional remarks. (1) The temperature at which a given tier of substates becomes blurred and can be averaged depends on the characteristic time of observation, t_{obs} . Motions that occur on a time scale much slower than t_{obs} cannot be seen. EF that are much faster than t_{obs} lead to a blurring of the substates. Specific heat measurements, for instance, are slow and have t_{obs} of the order of minutes. The characteristic time of ^{57}Fe Mössbauer effect is about 10^{-7} sec. X-Ray diffraction takes essentially instantaneous snapshots ($\sim 10^{-15}$ sec), and substates can be observed in the entire temperature range where diffraction data can be taken.^{19,21} (2) An EF can most likely not be described by a single relaxation time, but requires a distribution of times. Such a distribution implies that the barriers between substates span a range of energies.²⁹ (3) The discussion given above assumes that the times characteristic of the different EF are well separated. If the relaxation times of different tiers overlap, the situation becomes more complex.

In a protein with a hierarchical structure of the type shown in Fig. 3, the description of EF is relatively simple, but the characterization of FIMs, the functionally important motions, becomes rather complicated. A transition from one state to another, indicated by an arrow in Fig. 1, becomes a sequence of successive, and sometimes simultaneous, EF and FIMs.

The hierarchy of states and motions displayed in Fig. 3 suggests that proteins may have profound similarities with amorphous solids and glasses.^{30,31} It may therefore be possible to apply theories of glass relaxation to protein motions.⁴⁸

Role of Water

Water is essential for the function of proteins. As a typical example, Fig. 4 shows the enzymatic activity of lysozyme as a function of hydration, measured in grams of water per gram of protein.⁴⁹ At low hydration,

⁴⁸ R. G. Palmer, D. L. Stein, E. Abrahams, and P. W. Anderson, *Phys. Rev. Lett.* **53**, 958 (1984).

⁴⁹ P. H. Yang and J. A. Rupley, *Biochemistry* **18**, 2654 (1979).

FIG. 4. Enzymatic activity (□) and rotational relaxation rate (○) of an ESR probe as a function of hydration for lysozyme (after Rupley *et al.*⁵⁰).

the protein is inactive; the activity begins at about 0.2 g water/g protein. Also shown in Fig. 4 is the rotational relaxation rate of an ESR probe. Enzyme activity and relaxation rate show the same dependence on hydration, thus relating activity and dynamics to hydration.

Water is important in two respects. It forms a hydration layer around the protein with thermodynamic and dynamic properties that are different from bulk water.⁵⁰ Water molecules collide with the protein surface and exchange energy and momentum between protein and bath.⁵¹ Both of these features operate on globular proteins that are in the aqueous medium in the cell; the hydration shell is most likely also present for membrane proteins.

Hydration occurs in three steps,⁵²⁻⁵⁷ as sketched in Fig. 5. (1) Starting with a dehydrated protein, the first water molecules interact with charged groups. At neutral pH a number of negatively and positively charged groups are present on the protein surface. Myoglobin, a small globular

⁵⁰ J. A. Rupley, E. Gratton, and G. Careri, *Trends Biol. Sci.* **8**, 18 (1983).

⁵¹ E. Gratton, *Adv. Physiol. Sci.* **3**, 369 (1980).

⁵² G. Careri, A. Giansanti, and E. Gratton, *Biopolymers* **18**, 1187 (1979).

⁵³ G. Careri, E. Gratton, P. H. Yang, and J. A. Rupley, *Nature (London)* **284**, 572 (1980).

⁵⁴ I. D. Kunz and W. Kauzmann, *Adv. Protein Chem.* **28**, 239 (1973).

⁵⁵ B. Gavish, E. Gratton, and C. J. Hardy, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 750 (1983).

⁵⁶ R. Cooke and I. D. Kunz, *Annu. Rev. Biophys. Bioeng.* **3**, 95 (1974).

⁵⁷ J. L. Finney and P. L. Poole, *Comments Mol. Cell. Biophys.* **2**, 129 (1984).

FIG. 5. Schematic representation of the different hydration levels for a globular protein: hydration of charged groups, hydration of the protein backbone, and hydration of nonpolar groups on the protein surface.

protein, has about 40 charged groups at neutral pH. The interaction of water with a charge is characterized by a large free-energy and volume change.^{54,55} Water is electrostricted around negative and positive charges forming a hard, incompressible patch on the protein surface. At pH values corresponding to the pK of a given group, an equilibrium dynamic situation is established in which rapid localized energy and volume fluctuations occur. These fluctuations can be as large as the free energy of stabilization of the whole protein structure. The time scale of these events is on the order of microseconds to nanoseconds.⁵⁵ The hydration of charged groups is almost complete at 0.1 g water/g of protein corresponding to approximately two molecules of water per charge. (2) A second category of water molecules forms hydrogen bonds with the carbonyl and possibly with the N-H amide group of the protein backbone and with some of the side chains. The number of water molecules interacting with the backbone is quite large, and generally 70–80% of the backbone is involved. This extensive hydrogen-bonded network of water molecules has peculiar thermodynamic and dynamic properties. The specific heat of this water is different from that of the bulk solvent.⁵³ Also the resident time of this water is relatively long and the rotational properties are changed. Movements of the bound water molecules are limited by the formation and the breakage of hydrogen bonds, and this water is more immobilized than the

bulk solvent. The network of hydrogen-bonded water molecules on the protein surface forms patches. The lifetime of these structures can be quite short, on the order of nanoseconds to picoseconds, and can influence the dynamics of the substrate to which they are attached.⁵⁰ (3) A third category of water molecules forms well-defined structures around nonpolar residues owing to hydrophobic interactions. The current view is that transient water structures form around these groups, but the lifetime of these structures is quite short. The characteristics of the hydrophobic interaction, i.e., the entropic nature, must imply rather large fluctuations in conformation.

A fully hydrated protein has about 0.3–0.4 g water/g of protein, which corresponds to about 300 water molecules per protein (for Mb). The formation of the full hydration layer has a determinant role for the dynamics and for the physiological function of a protein. The effect on the dynamics is reflected in many properties, the specific heat, dielectric coefficient, NMR spectra, fluorescence depolarization, and others. As a general rule, at room temperature, a dehydrated protein is frozen in a given conformational substate. Also movements in the nanosecond time scale are blocked while motions in the picosecond time scale, corresponding to local vibrations and rotations, seem to persist. The mechanism for the effect of water on internal protein movements is not clear. The effect of the dielectric coefficient of water on protein charges is clearly not the sole cause for the change in microscopic interactions caused by water. Some peculiar characteristic interaction of water with the protein elements must be involved.⁵⁴

The study of the effect of hydration on the hierarchy of tiers sketched in the previous section would provide a better understanding of how water affects the EF of a protein and how FIMs can be triggered. It has been suggested that the cross-correlation between protein EF and EF of the bound water can be important for the existence of FIMs.⁵⁸ However, direct experimental evidence for the existence of a cross-correlation between protein motions and motions in the surrounding bath is not available, and it is not clear if the cross-correlation must be searched only among CS² tiers, as originally suggested.^{2,58}

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⁵⁸ G. Careri, in "Quantum Statistical Mechanics in the Natural Sciences" (B. Kursunoglu and S. L. Mintz, eds.). Plenum, New York, 1974.