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Concerted motions in copper plastocyanin and azurin: an essential dynamics study

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Abstract

Essential dynamics analysis of molecular dynamics simulation trajectories (1.1 ns) of two copper containing electron transfer proteins, plastocyanin and azurin, has been performed. The protein essential modes have been analysed in order to identify large concerted motions which could be relevant for the electron transfer function exerted by these proteins. The analysis, conducted for temporal windows of different lengths along the protein trajectories, shows a rapid convergence and indicates that for both the proteins the predominant internal motions occur in a subspace of only a few degrees of freedom. Moreover, it is found that for both the proteins the likely binding sites (i.e. the hydrophobic and negative patches) with the reaction partners move in a concerted fashion with a few structural regions far from the active site. Such results are discussed in connection with the possible involvement of large concerted motions in the recognition and binding interaction with physiological electron transfer partners. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Electron transfer proteins; Molecular dynamics; Essential dynamics; Concerted motions

1. Introduction

Plastocyanin (PC) and azurin (AZ), which are members of a family of proteins described collectively as blue-copper proteins [1] play a crucial role in acting as electron transfer (ET) agents in the photosynthetic and redox systems, respectively. Both the proteins consist of eight stranded β-strands, arranged in a β-sandwich, connected by random chains (turns) and of an α helical insertion. The PC and AZ copper reaction sites are at one end of the β-sandwich structures. The geometry of the PC copper site is described as a distorted tetrahedral arrangement of three strongly bound ligands (two histidine and one

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cysteine) and one more weakly bound ligand (methionine) around the copper [2]. The AZ copper site was found to share the same four ligands in a similar geometry, but with the possibility of a weak fifth ligand (glycine) [3].

The PC protein surface, which represents the barrier through which the electron has to enter in an ET event, is characterised by a pronounced negative patch (42–45 and 59–61 residues) and a flat hydrophobic patch (12, 33–36, 86, 89–90 residues) around the partly solvent exposed Cu-ligand His87 [4,5]; such patches being found to be involved in the binding interaction with cytochrome *f* and Photosystem I [4,5]. Similarly, on the AZ surface a large hydrophobic patch (13, 39, 42–44, 64, 72, 114–116, 119–120 residues) located around the Cu-ligand His117 has been suggested as a likely candidate for reaction of AZ with both nitrite reductase and cytochrome c_{551} [6].

Dynamic processes form an essential part of protein functionality and the knowledge of the atomic motions and their collective character in proteins is crucial for the understanding of their biological function [7]. In the ET copper proteins, small movements of protein residues near to the peculiar tetrahedral distorted copper reaction centre could modulate the value of the redox potential and the fine tuning of the ET reactions. It has been suggested that also movements of protein atoms far from the copper site could be relevant to allow the electron to travel a long distance from the docking site to the metal centre [8]. Generally, large-scale conformational transitions could be determined by relative and concerted movements between groups of protein atoms (e.g. small domains). Such types of motion could conceivably provide a wide range of protein conformations with small expenditure in energy. Each small domain would represent a structurally rigid entity composed of amino acids residues that move both cooperatively and coherently. In the ET copper-proteins, concerted motions between the binding sites and other different protein domains could be crucial for the partner recognition.

Recently, we have been strongly interested in the application of the molecular dynamics (MD) simulation approach to the investigation of the dynamical behaviour of PC and AZ in relationship to their functional role and a preliminary approach, based on the analysis of dynamical cross correlation matrix (DCCM), to identify protein correlated motions has been applied [9,10]. Such an analysis revealed the presence in both the PC and AZ proteins of correlated motions among residues that form the β-stranded protein scaffold and of correlated motions among protein regions likely involved in the ET mechanism [9,10]. However, as the cross-correlation analysis is not an appropriate method when the individual atom pairs fluctuate along orthogonal directions, a number of motions were counted as being totally uncorrelated [11,12]. Furthermore, such a method is not able to separate large-scale correlated fluctuations, which are likely candidates for functionally important motions, from small-scale random vibrations [11].

In the present paper, we exploit the capabilities offered by the application of the essential dynamics (ED) method in order to better identify the PC and AZ collective motions. The ED method [13] allows one to separate the large anharmonic motions (the essential subspace) in a MD trajectory from the small, Gaussian fluctuations (the near-constraints subspace). The ED procedure is similar to the multidimensional linear squares fit, applied for the first time by Garcia [14]. The motions in the essential subspace are found to be relevant for the biological function of several proteins [15–25]. However, if on one hand, it is assessed that a very small number of essential modes dominate protein motions [13], on the other, these dominant modes change from one sampling window to another. In addition, some authors [21,26,27] reported evidences of insufficient configurational sampling even in nanosecond scale simulations, suggesting that simulations could be unable to provide a reliable eigenvectors set. On the other hand, evidences that a few hundred picoseconds simulation is in general sufficient to obtain a reasonable convergence of the essential and near constraints subspace are also reported [18,28–31].

Here, we have performed MD simulations (1.1 ns), at 300 K, of fully hydrated PC [9] and AZ [10] and the ED analysis has been applied by sampling

temporal windows of different lengths (ranging from 100 to 800 ps) of the PC and AZ trajectories. Such an analysis has put into evidence that a rapid convergence occurs and that the predominant PC and AZ internal motions occur in a subspace of only a few degrees of freedom. In addition, the results have revealed, in both the proteins, the existence of correlated motions between the likely binding sites (i.e. the hydrophobic and negative patches) with the reaction partners and structural regions far from the active site.

2. Computational methods

MD simulations were carried out using GROMOS87 program [32] with the force field modified according to Mark et al. [33]. The X-ray crystal structures of poplar PC solved at 0.133 nm (1PCL entry of Brookhaven Protein Data Bank) [2] and of *Pseudomonas aeruginosa* AZ solved at 0.193 nm (4AZU entry of Brookhaven Protein Data Bank) [3] were used as the starting structures for the simulations. Since the GROMOS force field does not include parameters for amino acids liganded to metal ions, a modified force field has been employed. In particular, in both the PC and AZ simulations, a covalent bond between the copper and each ligand has been introduced to preserve the X-ray structure [9,10]. All the ionizable residues, with the exception of copper ligands, have been assumed to be in the ionized state corresponding to pH 6.0 and 5.5 of the PC and AZ crystals, respectively [2,3]. In both PC and AZ, the charge of cysteine liganded to copper has been set to -0.5e, while the other ligands have been considered neutral; the copper atom has been given a charge of 0.6e and 0.5e in PC and AZ, respectively. The total PC and AZ protein charges are then -7.9e and -3.0e, respectively.

The PC and AZ proteins were centered in truncated octahedron cells filled with 3514 and 3662 SPC/E waters, respectively. To avoid edge effects and to better describe the condition of full hydration, periodic boundary conditions have been applied. The simulations were performed at a temperature of 300 K and consisted of a total run of 1100 ps. Configurations of all trajectories and energy have been saved every 0.1 ps. Further details of the molecular dynamics protocols are presented elsewhere [9,10]. Evaluation of several geometric and energetic properties indicates that 100 and 300 ps were required for PC and AZ equilibrations, respectively. The last 800 ps of PC and AZ trajectories (i.e. from 300 to 1100 ps) were used for the MD and ED analyses.

Examinations of the molecular structures and analyses of the trajectories were carried out using the WHAT-IF modelling program [34] and the essential dynamics routines supplied therein.

To identify the correlated atomic motions of the two proteins, the MD trajectories of both PC and AZ were analysed according to the principal components or ED method [13,26,35]. This approach aims at identifying a new reference frame such that only a subset of coordinates, usually on the order of a few percent of the total, is sufficient to describe the overall dynamics of the system. The remaining degrees of freedom, corresponding to constrained harmonic oscillations, can be neglected. The ED method is based on the diagonalisation of the covariance matrix, built from the atomic fluctuations in a MD trajectory from which overall translational and rotational motions have been removed:

$$C_{ij} = \langle (X_i - X_{i,0})(X_j - X_{j,0}) \rangle \quad (1)$$

where X are the *x*-, *y*- and *z*-coordinates of the atoms fluctuating around their average positions X_0 and where $\langle \dots \rangle$ denote an average over time. Here, to construct the protein covariance matrices we have used C_α atom trajectories, including the copper atoms. Indeed, it has been shown that the C_α atoms contains all the information for a reasonable description of the protein large concerted motions [13]. Upon diagonalisation of the covariance matrix, a set of eigenvalues and eigenvectors is obtained. The eigenvectors of the covariance matrix correspond to directions in a $3N$ dimensional space (where N is the number of C_α atoms including the copper atom), and motions along single eigenvectors correspond to concerted fluctuations of atoms. The eigenvalues represent the total mean square fluctuation of the

system along the corresponding eigenvectors. If the eigenvectors are ordered according to decreasing eigenvalues, the first one describe the largest scale correlated motions, whereas the last one will correspond to small-amplitude vibrations [13]. By projecting all frames from the MD trajectory on an eigenvector a new trajectory can be generated which, upon visual inspection, reveals the correlated modes, e.g. the modes in which one part of protein tends to act concertedly with another.

3. Results and discussion

To assess the stability of the simulations and to check that the protein structures have properly equilibrated, a collection of dynamical properties as a function of the simulation time was monitored and reported in Table 1. The radii of gyration, R_g, of the proteins fluctuate around a mean value of 1.235 nm for PC and of 1.355 nm for AZ; such mean values being consistent with those expected for the hydrated proteins. The mean values of the potential and kinetic total energies of PC and AZ proteins are reported in Table 1; the time evolution of the potential and kinetic total energies reveals that, after a transient period of approximately 35 and 45 ps, respectively, both the energies are almost stable for both the proteins (data not shown).

The overall RMSD from crystal structures, which are found to be higher for the all protein atoms than for the C_α protein atoms, indicate a substantially slight displacement from the crystal structures during all the simulation run.

We have further analysed the MD trajectories of PC and AZ according to the ED approach. The plots of the PC and AZ eigenvalues, as calculated from Eq. (1), against the corresponding eigenvectors index for the first 50 modes of motion, at different trajectory lengths, are shown in Fig. 1a,b, respectively. At all temporal windows analysed a rapid decay in both the PC and AZ plots is observed. Such behaviour shows that, as also found for other proteins [13,15–25], a few eigenvectors suffice to describe the dynamics of both the proteins. The amount of motion associated to the subspace spanned by the eigenvectors can be defined as the corresponding subspace positional fluctuation where the eigenvalues are ordered in descending order [13]. The PC and AZ total positional fluctuations as a function of the increasing number of eigenvectors at different trajectory lengths are shown in Fig. 2a,b, respectively. The $3N$-dimensional configurational spaces formed by the PC and AZ C_α coordinates consist of 300 ($3NC_\alpha$; $N = 99$ residues and 1 Cu atom) and 387 ($3NC_\alpha$; $N = 128$ residues and 1 Cu atom),

Table 1
Selected properties of PC and AZ calculated from the MD simulated trajectories[a]

Parameter	Mean	S.D.	Min	Max	Drift
PC R_g (nm)	1.255	0.005	1.221	1.251	0.007
AZ R_g (nm)	1.355	0.004	1.342	1.372	$< 10^{-6}$
PC E_{pot} (MJ/mol)	−172.27	0.48	−170.41	−174.10	−0.31
AZ E_{pot} (MJ/mol)	−185.33	0.49	−187.21	−183.16	−0.48
PC E_{kin} (MJ/mol)	29.87	0.24	28.98	30.90	0.03
AZ E_{kin} (MJ/mol)	31.73	0.24	30.84	32.71	−0.02
PC R.M.S.D. of all atoms (nm)	0.174	0.011	0.139	0.201	0.020
AZ R.M.S.D. of all atoms (nm)	0.167	0.006	0.150	0.185	1×10^{-5}
PC R.M.S.D. of C_α atoms (nm)	0.141	0.012	0.104	0.169	0.020
AZ R.M.S.D. of C_α atoms (nm)	0.120	0.006	0.102	0.139	5×10^{-6}

[a]All PC and AZ values are calculated during the 300–1100 ps time interval. S.D., standard deviation; Min, minimal value; Max, maximal value; R.M.S.D., root mean square deviations; R_g, radius of gyration; E_{pot}, total potential energy; E_{kin}, total kinetic energy. The drift values are calculated from a linear regression and are given per picosecond.

respectively. Only a few eigenvectors are needed to describe the essential motions in both the two proteins as shown in Fig. 2, which shows the PC and AZ total positional fluctuations. Indeed, approximately 80% of the total motion is described by the first 30 eigenvectors for both the proteins. This means that most of the internal motions of PC and AZ can be represented by a subspace whose dimension is much smaller than original $3N$ configurational space.

In order to estimate the degree of anharmonicity of the motion along the first eigenvectors we have compared the probability distribution for the displacements along an eigenvector with an ideal Gaussian distribution derived from the amplitude (eigenvalue) of the corresponding eigenvector motion [13]. The sampling distribution functions for the PC and AZ displacements along

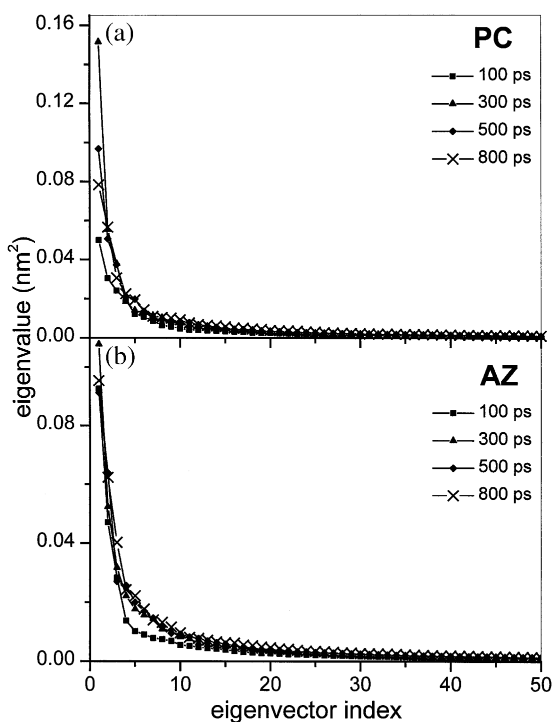

Fig. 1. Plots of PC (a) and AZ (b) eigenvalues against corresponding eigenvectors (sorted by decreasing eigenvalues) for the first 50 modes of motions. The PC and AZ C_α covariance matrices, calculated from Fig. 1, were constructed from simulations of different trajectory lengths.

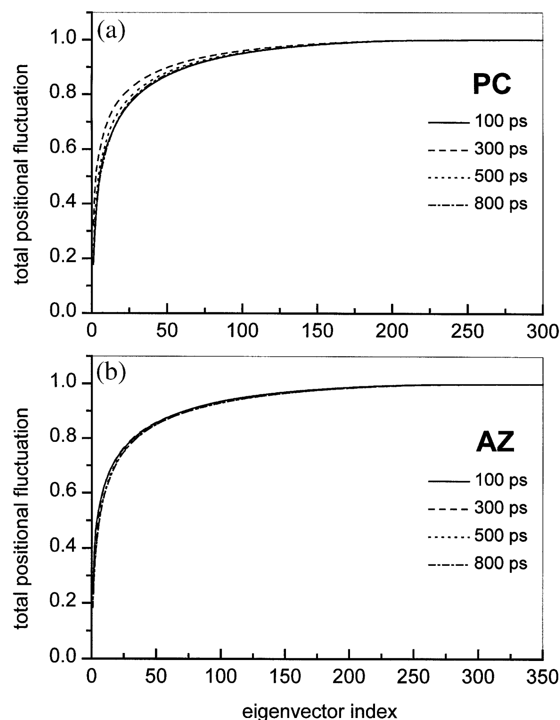

Fig. 2. Total positional PC (a) and AZ (b) fluctuations as a function of eigenvectors computed from the C_α covariance matrices constructed from simulations of different trajectory lengths.

the eigenvectors 1, 2, 4, 6, 10 and 50, as well as the corresponding Gaussian functions with the same covariance and average values are reported in inset of Fig. 3a,b, respectively. The comparison between the sampled and ideal Gaussian distributions yields a correlation coefficient, which can be plotted as a function of the eigenvector number [13,15]. Such correlation coefficients, at different PC and AZ trajectory lengths, are shown in Fig. 3a,b, respectively. We note that, at any time window analysed, after approximately the fifth eigenvector the position distributions of the motions along the eigenvectors start to be Gaussian (i.e. harmonic) for both the proteins. Indeed, from the fifth vector onward these correlation coefficients are higher than 0.95, indicating an approximately four-dimensional essential space. Therefore, as also illustrated in inset of Fig. 3, in PC and AZ

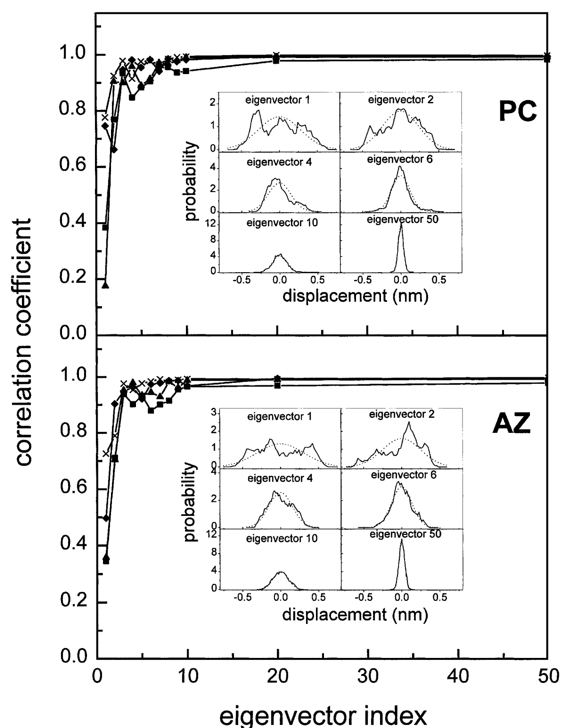

Fig. 3. Correlation coefficients between sampled eigenvector motions and ideal Gaussian distribution (derived from the eigenvalue of the corresponding eigenvectors) for the first 50 PC (a) and AZ (b) eigenvectors. Each curve represents a different length of the trajectory used in building the C_α covariance matrices: 100 ps (square); 300 ps (triangle); 500 ps (diamond); 800 ps (star). Inset: Probability distributions for the displacements along eigenvectors 1, 2, 4, 6, 10 and 50 obtained from the PC (a) and AZ (b) covariance matrices constructed over 800 ps simulation. Solid line: sampling distribution; dotted line: Gaussian distribution.

proteins the first four eigenvectors describe non-Gaussian, i.e. non-harmonic, fluctuations.

A similar result has been observed in other proteins where, indeed, the type of the motion along the first eigenvectors describing the protein essential subspaces has been found to be mainly anharmonic [13,15]. It should be remarked that the onset of anharmonic motions is crucial in order to activate the transition among the conformational substates, the sampling of which is relevant for the biological functionality [36]. Interestingly, the dimension of the essential spaces of PC and AZ, is smaller than those found for other proteins, e.g. lysozyme [13], thermolysin [15], protein-tyrosine phosphatase [21] and lipase [22] (approx. 10–15 essential eigenvectors). However, something similar was observed for the SH3 protein, which only has a binding function and for CRB protein, that is a simple carrier protein, whose essential spaces have been found to be represented by only approximately eight and four essential eigenvectors, respectively [16,17]. According to what hypothesised for these proteins, the small dimension of PC and AZ essential spaces seems to be correlated with the functional differences between these proteins. Indeed, lysozyme, thermolysin, lipase and protein-tyrosine phosphatase are proteins that catalyse an enzymatic reaction and therefore many more concerted motions could be required to perform such a complex task; on the contrary, the SH3, CRB, PC and AZ proteins are simple binding, carrier and ET proteins and a few essential motions to perform such functions may be enough.

The motion along any desired eigenvector can be visualised by projecting all trajectory frames onto a specific eigenvector; the new generated trajectory reveals, upon visual inspection, the motion in the direction defined by the eigenvector [13,15]. Here, the motions of PC and AZ along the first three eigenvectors have been investigated and the speed of convergence of their correlated modes has been analysed by sampling temporal windows of different lengths of both the PC and AZ trajectories. The absolute values of the components of the first three eigenvectors for PC and AZ, at different trajectory lengths, are shown as a function of residue number in Figs. 4–6. Generally, one would expect that with increasing simulation time a convergence of the fluctuations should occur and that similar features in the modes should be observed.

Indeed, a fast convergence of fluctuations is observed along the first eigenvector where relative large and similar concerted motions (> 0.10 nm) are found after 200 ps for PC, in correspondence of T1, T3, T5 turns and S6 β-strand (Fig. 4a) and after 400 ps for AZ, in correspondence of S2 β-strand and T4, T5 turns (Fig. 4b). A similar convergence trend as a function of the analysed trajectory length has been observed for lypase

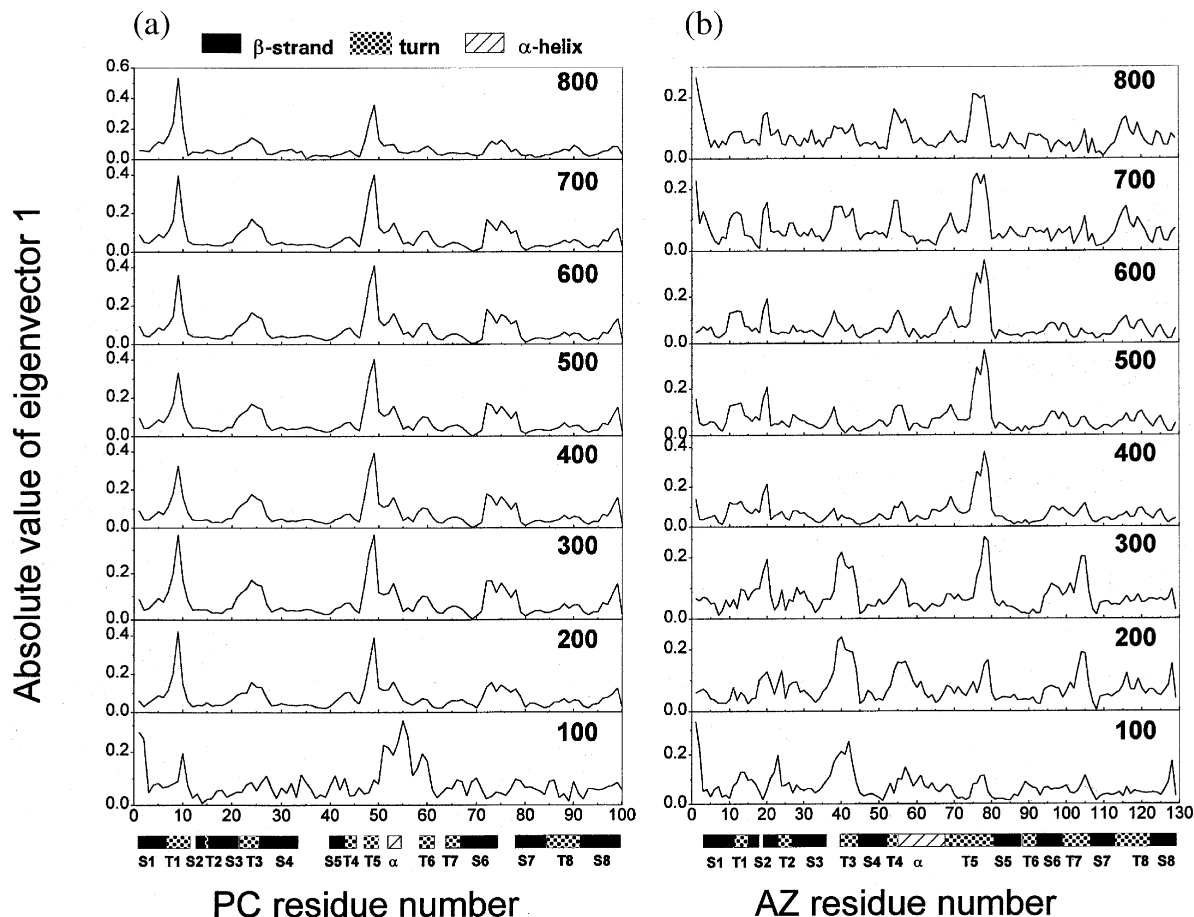

Fig. 4. Absolute values of the component of the first eigenvector obtained from the PC (a) and AZ (b) covariance matrices as a function of residue number. Each curve represents a different length of the trajectory used in the calculation (time intervals in ps are given to the right of the curves). Secondary structure elements are also shown.

protein [21]. For both PC and AZ, motions along eigenvector 2 and 3 vary with simulation time; however some fluctuations are conserved (Figs. 5 and 6). Indeed, as shown in Fig. 5a and Fig. 6a the fluctuation of T1 turn is conserved with the simulation time along both the second and third PC eigenvectors. Concerning the AZ concerted motions along the second and third eigenvectors, shown in Fig. 5b and Fig. 6b, a few structural regions, namely the T5 turn and N-terminal region, show conserved fluctuations.

A pictorial view of the PC and AZ motions along the first three eigenvectors of the 800-ps simulation C_α covariance matrices is shown in Fig. 7a,b, respectively. The analysis has been also extended to other PC and AZ trajectory lengths (i.e. 700, 600, 500-ps and so on) revealing a similar behaviour in term of concerted cluster of atoms (data not shown). Basically, the PC structure shows a rigid β-barrel core, formed by 8 β-strands (S), within which the copper reaction centre is located, and flexible connecting turn regions (T). As shown in Fig. 7a, a few regions of the PC protein, mainly turn regions, appear to be involved in concerted motions. In particular, the motion along the first eigenvector is concentrated in four structural regions, namely the T1, T3, T5 turns (7–12, 20–25 and 47–50 residues, respec-

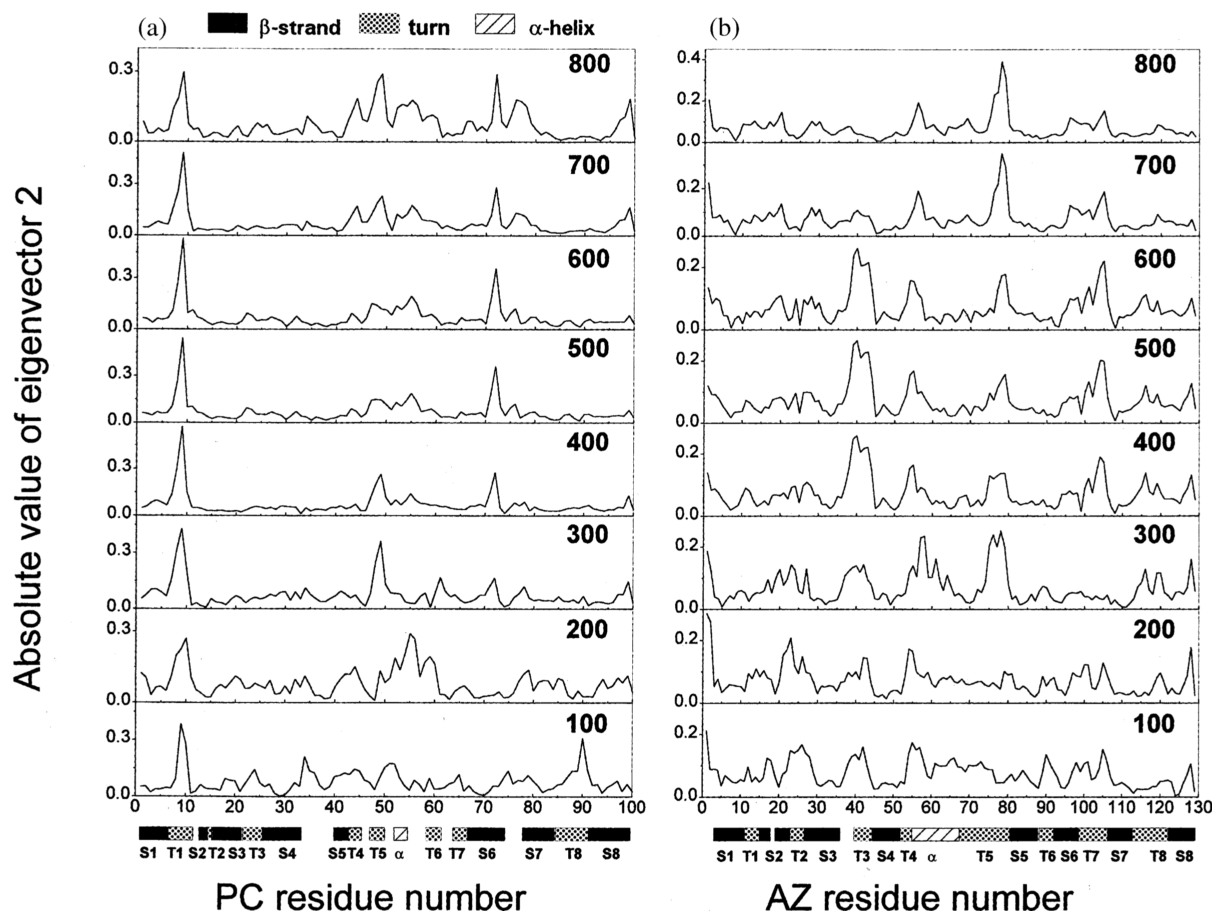

Fig. 5. Absolute value of the component of the second eigenvector obtained from the PC (a) and AZ (b) covariance matrices as a function of residue number. Each curve represents a different length of the trajectory used in the calculation (time intervals in ps are given to the right of the curves). Secondary structure elements are also shown.

tively) and S6 β-strand (72–75 residues) which move concertedly. It is interesting to note that the Leu12 residue, that is a highly conserved residue, has been found to be crucial for the binding recognition with the physiological reaction partner [4,37]. The PC motion along the second eigenvector shows that the hydrophobic (7–12 and 34–36 residues) and negative (43–45 residues) patches move concertedly with other structural regions, namely the α-helix, T5 turn and S6, S7 and S8 strands. Such a concerted motion concerns the binding site movements that could be necessary to perform the binding recognition via hydrophobic and negative interactions. Similarly, the motion along the third eigenvector concerns the concerted motions of the functional T1 and T6 turns, containing the 7–12 and 60–62 residues which are located in the hydrophobic and negative patches, and the α-helix, T3 and T5 turns and S6, S7 and S8 β-strands. It should be remarked that the binding and protein docking (i.e. between PC and its physiological partner) are thought be dynamical processes during which configurational fluctuations and structural rearrangements in the proteins occur [38,39].

In this respect, concerted motions of the likely

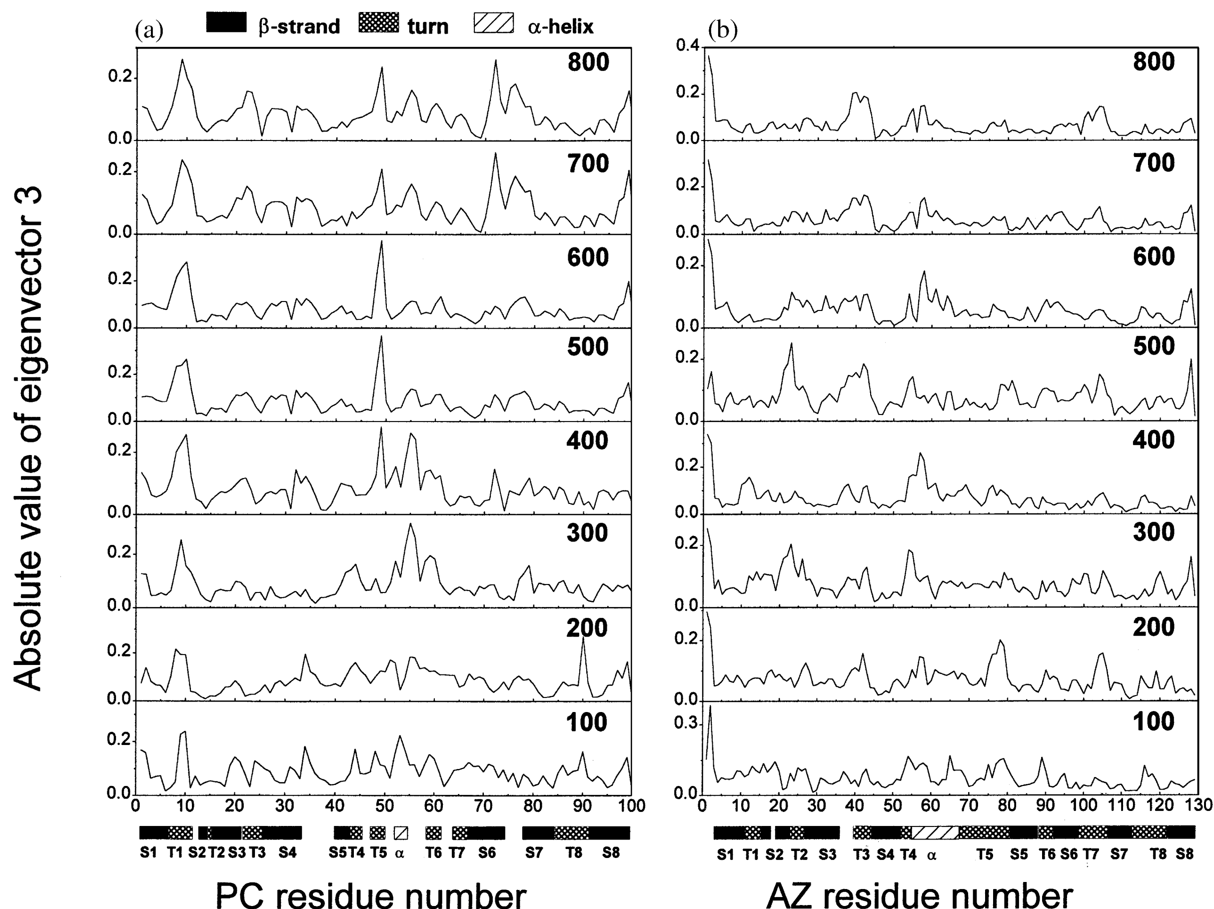

Fig. 6. Absolute value of the component of the third eigenvector obtained from the PC (a) and AZ (b) covariance matrices as a function of residue number. Each curve represents a different length of the trajectory used in the calculation (time intervals in ps are given to the right of the curves). Secondary structure elements are also shown.

binding protein structures could represent functional fluctuations related to the binding recognition between PC its physiological ET partner.

In Fig. 7b the AZ concerted motions along the first three eigenvectors are shown. Similarly to PC, the AZ structure is characterised by a rigid core formed by the β-barrel, flexible turn loops (T) connecting the β-strands (S) and by an extra flap, containing the only α-helix on the outside of the β-barrel. In addition, a disulphide bridge connecting the S1 and S3 β-strands (Cys3–Cys26) at 2.6 nm from the copper-binding site [3], is also present.

Analysis of the motions along all the three eigenvectors reveals that the presence of such disulphide bridge dictates a sort of rigidity to the structural regions surrounding it; on the contrary, a flexible region located around the α-helix is observed. In particular, motion along the first eigenvector shows that α-helix, the N-terminal and the 18–22 residues move in a concerted way with the T3, T8 and T5 turn regions, which contain the hydrophobic patch. Such concerted modes could play a crucial role in facilitating the binding interaction with the physiological reaction partners; such binding being supposed to be mediated by the hydrophobic patch [5,6]. Motion along the second eigenvector is mainly concentrated in the

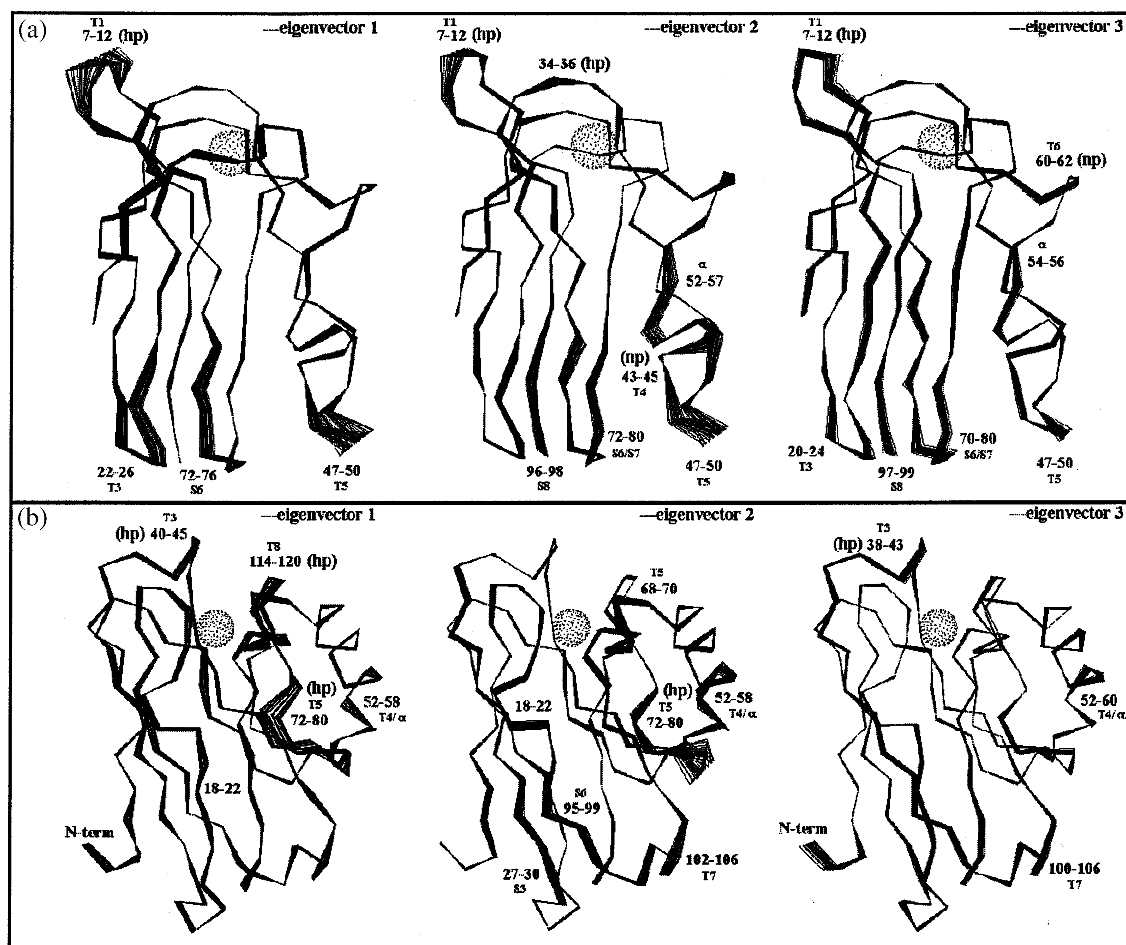

Fig. 7. Twenty-five frames taken at equally spaced intervals from the motions along the first three eigenvectors of the PC (a) and AZ (b) covariance matrices constructed over 800-ps simulation. The structural regions, the residue numbers, the negative (np) and hydrophobic (hp) patches are also indicated.

flap region, containing the 52–58 and 60–70 residues, the hydrophobic 72–80 residues, the T7 turn, the S3 and S6 β-strands and the 18–22 residues, which move concertedly. A few regions appear to be involved in concerted motion along the third eigenvector. In particular, the hydrophobic T3 turn, which lies near to the copper reaction centre, move concertedly with the α-helix, the T7 turn and the N-terminal region, which are regions far from the reaction centre.

The overall emerging picture is that, in both the PC and AZ proteins, the functional regions, i.e. the likely binding sites, which are close in the tertiary structure to the copper site, move in a concerted fashion with structural regions, mainly turns, that are far from the reaction centre. Something similar was found in our previous MD simulation studies of PC and AZ, where a preliminary approach, based on the analysis of DCCM, to extract protein correlated motions was applied [9,10]. Indeed, we found that all the secondary structure elements in both the proteins moved approximately in a concerted fashion and that correlated motions between turn regions which are putatively implied in the ET process occurred [9,10].

However, by comparing the DCCM results with those obtained by ED analysis, we observe that a number of ED concerted motions are not found in DCCMs of PC and AZ. In this respect, it should be remarked that in DCCM analysis when two atoms move exactly in phase and with the same period, but along perpendicular lines they will have a DCCM correlation value of zero, which means that a number of motions are counted as totally uncorrelated [11,12]. In addition, the correlation coefficients obtained from the DCCM method do not bear any information about the magnitude of the motions; therefore, it may happen that both small- and large-scale collective motions were expressed by the same correlation coefficient [11,12].

Actually, it is desirable to separate large-scale correlated fluctuations, which are, as already mentioned, likely candidates for functionally important motions, from small-scale random vibrations. In this respect, ED method has proved to be an appropriate method.

4. Conclusion

The ED analysis at different PC and AZ trajectory lengths has shown that the predominant internal motions in both the proteins occur in a subspace of only a few degrees of freedom and that a number of regions in both the two proteins move in a concerted fashion. In particular, PC and AZ essential modes converge after 200 and 400 ps, respectively, and only small changes are observed, along those particular essential modes, with increasing the simulation time. A detailed analysis, supported also by three-dimensional analysis of the trajectories, has revealed, in both the proteins, the existence of correlated motions between the likely binding sites (i.e. the hydrophobic and negative patches) with the reaction partners and a few structural regions far from the active site. Such a finding suggests that large concerted movements of protein regions remote from the copper site with protein portions close to the copper reaction centre, could play a important role for the binding recognition between PC and AZ and their physiological ET partners.

References

[1] E.T. Adman, in: Topics in molecular and structural biology: metalloproteins, ed. P.M. Harrison, Structure and Function of Small Blue Copper Proteins, Vol. 6, part I (Chemie Verlag, Weinheim, 1985) pp. 1–42.

[2] J.M. Guss, H.D. Bartunik, H.C. Freeman, Accuracy and precision in protein structure analysis: restrained least-squares refinement of the structure of poplar plastocyanin at 1.33 Å resolution, Acta Cryst. B48 (1992) 790–811.

[3] H. Nar, A. Messerschmidt, R. Huber, M. van de Kamp, G.W. Canters, X-Ray crystal structure of two site-specific mutants His35Gln and His35Leu of azurin from *Pseudomonas aeruginosa*, J. Mol. Biol. 218 (1991) 427–447.

[4] M.R. Redinbo, T.O. Yeates, S. Merchant, Plastocyanin: structural and functional analysis, J. Bioener. Biomembr. 26 (1994) 49–66.

[5] O. Farver, Y. Shahak, I. Pecht, Electron uptake and delivery sites on plastocyanin in its reaction with the photosynthetic electron transport system, Biochemistry 21 (1982) 1885–1890.

[6] M. van de Kamp, M.C. Silvestrini, M. Brunori, J.V. Beeumen, F.C. Hali, G.W. Canters, Involvement of the hydrophobic patch of azurin in the electron transfer reactions with cytochrome *c*, Eur. J. Biochem. 194 (1990) 109–118.

[7] R. Huber, W.S. Bennet, Functional significance of flexibility in proteins, Biopolymers 22 (1983) 261–279.

[8] D.N. Beratan, J.N. Betts, J.N. Onuchic, Protein electron transfer rates set by the bridging secondary and tertiary structure, Science 252 (1991) 1285–1288.

[9] A. Ciocchetti, A.R. Bizzarri, S. Cannistraro, Long-term molecular dynamics simulation of copper plastocyanin in water, Biophys. Chem. 69 (1997) 185–198.

[10] C. Arcangeli, A.R. Bizzarri, S. Cannistraro, Long-term molecular dynamics simulation of copper azurin: structure, dynamics and functionality, Biophys. Chem. 78 (1999) 247–257.

[11] I. Ichiye, M. Karplus, Collective motions in proteins; a covariance analysis of atomic fluctuations in molecular dynamics and normal modes simulations, Proteins: Struct. Funct. Genet. 11 (1991) 205–217.

[12] P.H. Hünenberger, A.E. Mark, W.F. van Gunsteren, Fluctuation and cross-correlation analysis of protein motions observed in nanosecond molecular dynamics simulations, J. Mol. Biol. 252 (1995) 492–503.

[13] A. Amadei, A.B.M. Linssen, H.J.C. Berendsen, Essential dynamics of proteins, Proteins: Struct. Funct. Genet. 17 (1993) 412–425.

[14] A.E. Garcia, Large-amplitude nonlinear motions in proteins, Phys. Rev. Lett. 68 (1992) 2696–2699.

[15] D.M.F. van Aalten, A. Amadei, A.B.M. Linsenn, V.G.H. Eijsink, G. Vriend, H.J.C. Berendsen, The essential dynamics of thermolysin: confirmation of the hinge-bend-

ing motion and comparison of simulations in vacuum and water, Proteins: Struct. Funct. Genet. 22 (1995) 45–54.
[16] D.M.F. van Aalten, J.B.C. Findlay, A. Amadei, H.J.C. Berendsen, Essential dynamics of the cellular retinol-binding protein — evidence for ligand-induced conformational changes, Prot. Engng. 8 (1995) 1129–1135.
[17] D.M.F. van Aalten, A. Amadei, R. Bywater, J.B.C. Findlay, H.J.C. Berendsen, C. Sander, P.F.W. Stouten, A comparison of structural and dynamic properties of different simulation methods applied to SH3, Biophys. J. 70 (1996) 684–692.
[18] B.L. de Groot, D.M.F. van Aalten, A. Amadei, H.J.C. Berendsen, The consistency of large concerted motions in proteins in molecular dynamics simulations, Biophys. J. 71 (1996) 1707–1713.
[19] G.H. Peters, D.M.F. van Aalten, O. Edholm, S. Toxvaerd, R.P. Bywater, Dynamics of proteins in different solvent systems: analysis of essential motion in lipases, Biophys. J. 71 (1996) 2245–2255.
[20] D.M.F. van Aalten, W.D. Hoff, J.B.C. Findlay, W. Crielaard, K.J. Hellingwerf, Concerted motions in the photoactive yellow protein, Prot. Engng. 11 (1998) 873–879.
[21] G.H. Peters, R.P. Bywater, Computational analysis of chain flexibility and fluctuations in *Rhizomucor miehei* lipase, Prot. Engng. 12 (1999) 747–754.
[22] G.H. Peters, T.M. Frimurer, J.N. Andersen, O.H. Olsen, Molecular dynamics simulations of protein-tyrosine phosphatase 1B. I. Ligand-induced changes in the protein motions, Biophys. J. 77 (1999) 505–515.
[23] L. Stella, E.E. Di Iorio, M. Nicotra, G. Ricci, Molecular dynamics simulations of human glutathione transferase P1-1: conformational fluctuations of the apo-structure, Proteins: Struct. Funct. Genet. 37 (1999) 10–19.
[24] L.M. Horstink, R. Abseher, M. Nilges, C.W. Hilbers, Functionally important correlated motions in the single-stranded DNA-binding protein encoded by filamentous phage Pf3, J. Molec. Biol. 287 (1999) 569–577.
[25] P.L. Chau, D.M.F. van Aalten, R.P. Bywater, J.B.C. Findlay, Functional conceted motions in the bovine serum retinol-binding protein, J. Comput. Aided Molec. Des. 13 (1999) 11–20.
[26] M.A. Balsera, W. Wriggers, Y. Oono, K. Schulten, Principal component analysis and long time protein dynamics, J. Phys. Chem. 100 (1996) 2567–2572.
[27] J.B. Clarage, T. Romo, B.K. Andrews, B.M. Pettitt, G.N. Phillips, Jr., A sampling problem in the molecular dynamics simulations of macromolecules, Proc. Natl. Acad. Sci. USA 92 (1995) 3288–3292.
[28] A. Amadei, M.A. Ceruso, A. Di Nola, On the convergence of the conformational coordinates basis set obtained by the essential dynamics analysis of proteins' molecular dynamics simulations, Proteins: Struct. Funct. Genet. 36 (1999) 419–424.
[29] A. Amadei, A.B.M. Linssen, B.L. de Groot, D.M.F. va Aalten, H.J.C. Berendsen, An efficient method for sampling the essential subspace of proteins, J. Biomol. Struct. Dyn. 13 (1996) 615–625.
[30] B.L. de Groot, A. Amadei, R.M. Scheek, N.A.J. van Nuland, H.J.C. Berendsen, An extended sampling of the configurational space of HPr from *E. coli*, Proteins: Struct. Funct. Genet. 26 (1996) 314–322.
[31] D.M.F. van Aalten, B.L. de Groot, J.B.C. Findlay, H.J.C. Berendsen, A. Amadei, A comparison of techniques for calculating protein essential dynamics, J. Comp. Chem. 18 (1997) 169–181.
[32] W.F. van Gunsteren and H.J.C. Berendsen, Groningen Molecular Simulation (GROMOS) Library Manual (Biomos, Groningen, 1987).
[33] A.E. Mark, S.P. Helden, P.E. Smith, L.H.M. Janssen, W.F. van Gunsteren, Convergence properties of free energy calculations: α-cyclodextrin complexes as a case study, J. Am. Chem. Soc. 116 (1994) 6293–6302.
[34] G. Vriend, WHAT IF-A molecular modeling and drug design program, J. Mol. Graph. 8 (1990) 52–56.
[35] A.E. Garcia, G. Hummer, Conformational dynamics of cytochrome *c*: correlation to hydrogen exchange, Proteins: Struct. Funct. Genet. 36 (1999) 175–191.
[36] H. Frauenfelder, F. Parak, R.D. Young, Conformational substates in proteins, Annu. Rev. Biophys. Biophys. Chem. 17 (1988) 451–479.
[37] K. Sigfridsson, S. Young, Ö. Hansson, Structural dynamics in the plastocyanin–photosystem I electron-transfer complex as revealed by mutant studies, Biochemistry 35 (1996) 1249–1257.
[38] G.M. Ullmann, E-W. Knapp, N.M. Kostić, Computational simulation and analysis of dynamic association between plastocyanin and cytochrome *f*. Consequences for the electron-transfer reaction, J. Am. Chem. Soc. 119 (1997) 42–52.
[39] E.V. Pletneva, D.B. Fulton, T. Kohzuma, N.M. Kostić, Protein docking and gated electron-transfer reactions between zinc cytochrome c and the new plastocyanin from the Fern *Dryopteris crassirhizoma*. Direct kinetic evidence for multiple binary complexes, J. Am. Chem. Soc. 122 (2000) 1034–1046.