Eur Biophys J (1998) 27: 183–196

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ARTICLE

P. L. San Biagio · D. Bulone · V. Martorana
M. B. Palma-Vittorelli · M. U. Palma

Physics and biophysics of solvent induced forces: hydrophobic interactions and context-dependent hydration

Received: 22 July 1996 / Accepted: 5 December 1997

Abstract Solvent induced forces (SIFs) among solutes derive from solvent structural modification due to solutes, and consequent thermodynamic drive towards minimization of related free energy costs. The role of SIFs in biomolecular conformation and function is appreciated by observing that typical SIF values fall within the 20–200 pN interval, and that proteins are stable by only a few kcal mol^{-1} (1 kcal mol^{-1} corresponds to 70 pN Å). Here we study SIFs, in systems of increasing complexity, using Molecular Dynamics (MD) simulations which give time- and space-resolved details on the biologically significant scale of single protein residues and sidechains. Of particular biological relevance among our results are a strong modulability of hydrophobic SIFs by electric charges and the dependence of this modulability upon charge sign. More generally, the present results extend our understanding of the recently reported strong context-dependence of SIFs and the related potential of mean force (PMF). This context-dependence can be strong enough to propagate (by relay action) along a composite solute, and to reverse SIFs acting on a given element, relative to expectations based on its specific character (hydrophobic/ philic, charged). High specificity such as that of SIFs highlighted by the present results is of course central to biological function. Biological implications of the present results cover issues such as biomolecular functional interactions and folding (including chaperoning and pathological conformational changes), coagulation, molecular recognition, effects of phosphorylation and more.

Key words Protein folding/unfolding · Charge-apolar solute interaction · Molecular dynamics · Protein coagulation · Detergents

P. L. San Biagio · D. Bulone · V. Martorana
M. B. Palma-Vittorelli · M. U. Palma (✉)
INFM and Department of Physical and Astronomical Sciences,
University of Palermo, Via Archirafi 36, I-90123 Palermo, Italy

P. L. San Biagio · D. Bulone · V. Martorana
CNR Institute for Interdisciplinary Applications of Physics,
Via Archirafi 36, I-90123 Palermo, Italy

Introduction

Hydration and associated free energy G_{SW} are familiar concepts in solution thermodynamics. Microscopically, they are the results of structural modifications of the aqueous solvent due to solutes. The thermodynamic drive towards minimization of G_{SW} is expressed by solvent induced forces (SIFs), written as the negative gradient of G_{SW} with respect to coordinates of the given solute. A subset of solvent induced interactions is that of "hydrophobic interactions", taking place among apolar solutes in the aqueous solvent (Frank and Evans 1945). Since long ago hydrophobic interactions have been thought to play a central role in biomolecular conformation and function (Kauzmann 1959). The idea has generated innumerable publications (see e.g. Tanford 1980; Ben Naim 1994; Dill et al. 1995) Hydrophobic interactions and, in general, solvent-induced interactions among solutes of any kind have usually been treated in terms of pair additivity, that is disregarding higher order interactions (for biological relevant cases, see e.g. Makatadze and Privalov 1995; see, however, also Lazaridis et al. 1995). High order interactions have been mostly considered in their average effects only, e.g. by means of virial expansion. In views of this type, notions such as "hydrophilic" and "hydrophobic" rest on solid experimental grounds, although hydrophobicity scales may become questionable as a consequence of triple and quadruple correlations of solute interactions (Ben Naim 1990 a and b). In fact, in the case of proteins, approaches in terms of average hydrophobic interactions have proved very useful down to a scale encompassing several residues. This is the case, for example in "hydrophobic collapse", a useful concept in a somewhat coarse grained description of protein folding (Dill et al., 1995). It is also the case in the derivation of the role of the solvent in the functional ΔG (T–R) free energy difference between T and R conformations of hemoglobin (HbA). In this case the work due to hydrophobic forces offsets that of all other forces and is crucial to the function of HbA, the paradigm of regulatory enzymes (Bulone et al. 1991, 1992 and 1993; Palma et al 1994).

Successful treatments such as the ones above make it clear that the energetics of solvent induced interactions often covers a crucial role in biomolecular conformation and functional interactions. This is, after all, not surprising if we recall that proteins are stable by only a few kcal mol^{-1} (Privalov and Gill 1988), which is equivalent to the work of a typical SIF (20–200 pN) across a few Angstrom or less (1 kcal mol^{-1} being equivalent to 70 pN Å). However, for our understanding of protein function on the grounds of basic physical laws, accounting for overall energetics is only a prerequisite. Indeed, the frequently encountered high specificity of intra- and inter-biomolecular interactions often depends on exquisitely specific molecular recognition through the solvent and on equally specific functional interactions. These interactions concern a small number of very close residues of different types (apolar, polar, charged), which may be parts of the same protein or of different functionally interacting biomolecules. In these conditions, concepts derived from the field of diluted or semi-diluted solutions or, more broadly, implying averagings of some type, cannot be taken for granted without further scrutiny. This is because ignoring interactions of order higher than two, or at most averaging them out, can easily cancel specific and functionally all-important details.

This concept is crucial to the present work. In order to illustrate it, let us call $G_{SW}(1)$ the hydration free energy of an isolated solute. In the case of n solutes at large distances (infinite dilution) the total hydration free energy will be $\Sigma_i^n G_{SW}(i)$, that is the sum of n independent G_{SW} single solute terms. However, this is no longer true when the solute distance allows the occurrence of overlaps of hydration regions of single solutes, as in the case of protein residues (see e.g. Lifson and Oppenheim 1960). In such cases the total hydration free energy is expected (and indeed found) to exhibit a strong many body character (Palma et al. 1994; Brugé et al. 1996 a; Martorana et al. 1996 and 1997), so that:

$$G_{SW}(1,2,\ldots,n) = \sum_{i}^{n} G_{SW}^{(i)}(i) + \sum_{i<j}^{n} \delta G_{SW}^{(2)}(i,j) + \ldots + \sum_{i<j<\ldots}^{n} \delta G_{SW}^{(k)}(i,j,\ldots) + \delta G_{SW}^{(2)}(i,j,\ldots,n) \qquad [1]$$

Here the δG_{SW} terms of different order represent contributions of pair, triplet, . . . n-plet interactions among solutes in establishing (self-consistently and collectively) the total hydration free-energy. These terms will depend upon distance and mutual configuration of all elements. As already mentioned, the negative gradient of G_{SW} with respect to coordinates of a given solute expresses the solvent-induced force (SIF) acting on that solute, first derived, although in a different formulation, by Kirkwood (1935). We note that, in principle, Eq. (1) shows that a change of type or position of even one element affects δG_{SW} terms of all orders and, consequently, the total G_{SW} and related SIFs on any other element. We further note from Eq. (1) that the existence of SIFs requires at least the existence of the first order $\delta G_{SW}^{(2)}$ terms, while their non-additivity requires higher order δG_{SW} terms. Since the latter have the same origin as SIFs themselves, they are expected and indeed found to be of comparable magnitude.

The many body character of solute-solvent interactions, as detailed in Eq. (1) has been shown by MD studies to have unexpectedly strong and even counter-intuitive effects. These include a strong non pair additivity of SIFs (Brugé et al. 1996a; Martorana et al. 1997), an equally strong context-dependence and long range correlated or relayed action (Martorana et al. 1996 and 1997), and even a sign reversal of SIFs on specific protein residues, relative to predictions based on their individual (hydrophobic/philic) character (Martorana, Corongiu and Palma, to be published). This shows that simple and useful concepts such as hydrophobic/philic are not neccessarily sufficient for the treatment of solvent-induced interactions in the complex context of proteins. Consequently, we must be ready to deal with a higher degree of complexity.

The main concern of this paper is a study of solvent induced interactions among apolar and charged solute elements and among solely apolar ones, at distances comparable with those within functional groups of residues in a protein. With the aim of eliciting the full size and significance of high order interactions, we make no use of truncations or of averagings of any kind and perform molecular dynamics (MD) simulations taking solvent molecules explicitly into account. This allows us to obtain space and time resolved details which are not jointly accessible to laboratory experiments or to computer efficients approximate methods.

In what follows we recall some results and report new and extensive data on SIFs acting between hydrophobic and electrically charged solutes. We demonstrate for the first time the strong and biologically very significant modulation of pairwise hydrophobic interactions by a third (otherwise identical) solute bearing a positive, negative and null electric charge. We also elicit and discuss the microscopic and quantitative origins of these novel, strongly collective effects. Use of simple model solutes avoids ambiguities in tracing such origins. Aspects strictly related to the particulate nature of the solvent are highlighted. Time-resolved and space-resolved features are also presented and related to hydration, to the dynamics of hydration water, and to the known spectroscopic properties of bulk water. Finally, we proceed to the study of solutes of increasing complexity, up to the realistic case of a protein. Significant biological implications of the present results are discussed in closing. In an Appendix we use the concept of configurational energy landscape and related "inherent structures" to express hydration and related free energy. This allows a straightforward visualization and a quantitative expression of hydration free energy and forces.

A summary of basic concepts

The known complexity of liquid water can be traced to the multitude of geometrically and topologically distinct configuration of its hydrogen-bond network. This multitude is reflected, for example, in its unusually large contribution to specific heat (Eisenberg and Kauzmann, 1969; Kell, 1972). In the absence of solutes, as a result of frequent switching among different statistically populated configurations, the probability of finding water molecules in any elementary volume of the fluid is uniformly distributed (uniform density). Solutes alter the statistically populated water configurations, as combined result of excluded volume and interaction potential. The consequent change of the configurational energies and multiplicity causes related changes of enthalpy, entropy and free energy. The latter, G_{SW}, is the hydration free energy. This and the corresponding alteration of configurational lifetimes and of spatial statistical distribution of solvent molecules (non uniform local density around solutes) characterize the thermodynamics and the spatial and dynamic features of hydration. Each solute (or each element such as protein residues), at any given time, is subject to a force F_S which is the vectorial sum of all forces F_{Si} exerted by each solvent molecule, through the appropriate interaction potential. The average SIF in expressed as:

$$SIF = < F_S > = < \sum_i F_{Si} > \quad [2]$$

where the sum is carried out over all solvent molecules and the angular brackets express thermodynamic averaging over all solvent configurations (Kirkwood 1935). Clearly, in the case of a single symmetric solute all forces will average to zero. Asymmetries in the statistical distribution of water molecules (non-uniform density), jointly caused by the given solute and by other near-by solutes produce non-zero average SIFs. This shows the causal relation between hydration and SIFs and provides a useful view of the latter, in terms of space resolved contributions (Brugé et al. 1996, a). In fact, each elementary volume will contribute a force obtained from the appropriate interaction potential, weighted by the statistical occupancy propability of solvent molecules (as collectively perturbed by all solutes). The resulting SIF is the non-zero difference of opposite (comparatively much larger) contributions. Also, time-resolved features of the instantaneous F_s (which we have averaged out in Eq. (2)) reflect the dynamic properties of hydration water.

SIFs can be expressed either as per Eq. (2), or as the gradient of the hydration free energy (Eq. (1)). Alternatively, one can define a potential of mean force, PMF (Kirkwood 1935; Hill 1956), such that its negative gradient (in the absence of direct solute-solute force) is SIF. As G_{SW}, PMF is of course a many-body potential. Accordingly, analytical treatments based on Eq. (2) require the use of correlation functions of all orders (see Appendix), which turns out to be a formidable task. To make it manageable, Kirkwood introduced the superposition approximation (KSA). In this approximation (Kirkwood 1935; Hill 1956), factorizability of higher order correlation functions in terms of pair correlation functions is assumed. In this way, only the term responsible for the existence of SIFs is retained, not those responsible for non pair additivity. In terms of Eq. (1), KSA is equivalent to neglecting all δG_{SW} terms for three and more particles, responsible for the many body character of hydration and of PMF, and for non pair additivity of SIFs. Further developments and applications to complex systems of Kirkwood's theory had to wait for the availability of modern computing facilities and adequate interaction potentials (Stillinger and Rahman 1974; Matsuoka et al. 1976; Jorgensen 1979; Mc Donald et al. 1980; Clementi et al. 1981). Treatments such as Molecular Dynamics (MD) simulations, however, remain very demanding in terms of computing time when dealing with large systems. This has stimulated the advent of a number of computing efficient methods retaining KSA or similar approximations. Such highly efficient approaches using approximations of various types have been tested in a number of cases and shown to reproduce with reasonable adequacy features of free energies and hydration (see e.g. Hirata et al. 1982; Pettitt et al. 1986; Kitao et al. 1991; Klement et al. 1991; Pellegrini and Doniach 1995; Garde et al. 1996). This is because non additivities are less likely to show up in cases involving averages of large numbers of interactions (de Gennes 1979) than in cases involving differences of large terms (Lazaridis et al. 1995). Interestingly, the adequacy of approximated methods to the coarse-grain understanding of mesoscopic features and phenomena is indorsed by both experimental and theoretical work (Martorana et al. 1997). However, those methods are no longer adequate when the main concern is to understand, in terms of basic physical laws, single, microscopic situations (say, at the biologically significant single residue level) and the physical drive governing related, local events. In these cases accuracies of, say, 10% or better in computing, for example, hydration are in general far from sufficient. Indeed, as we shall see in what follows, a fully explicit and non approximate treatment is required in order to understand crucial detailed features on which the exquisite functional specificity of biomolecules is based (Palma-Vittorelli et al. 1993; Palma et al. 1994; Brugé et al. 1994; Smith and Pettitt 1994).

Methods

Long MD simulations (up to 1,200 ps) were performed in the microcanonical (NVE) ensemble, at T=298 °K, and density 1 gr cm^{-3}, using a modified (leap-frog) Verlet algorithm (Corongiu and Martorana 1993). Periodic boundaries were used, and long-range electrostatic forces were dealt with by Ewald sums, notwithstanding their high computing cost. For water-water interactions we used the TIP4P potential (Jorgensen et al. 1983), particularly appropriate in the case of ionic solutions (Jorgensen et al. 1993; Del Buono et al. 1994). We used hydrophobic "solutes", modeled as Lennard Jones (LJ) spheres having the same potential parameters as in TIP4P ($\varepsilon = 0.64875$ KJ mol^{-1} and $\sigma = 3.154$ Å). When appropriate, a unitary electronic charge of either sign was attributed to one of the LJ solutes, and neutralized by an evenly distributed charge density of opposite sign (Figueirido et al. 1995). Solutes were kept in fixed positions, in the configurations specified below, in a bath of water molecules. The simulation box was a rectangular parallelepiped. Its size was chosen for each solute configuration so as to allow at least three layers of water molecules between solutes and boundaries. Accordingly, the total number of molecules (water+solutes) ranged, for different cases, from 343 to 728. Independent runs of not less than 800 and up to 1,200 ps were obtained for each configuration of solutes. For better statistical significance, each run was obtained as a sequence of 200 ps trajectories decoupled from each other by 20 ps annealings at 800 °K, followed by 40 ps equilibration at 298 °K. SIFs on each solute could by computed as time averages of instantaneous force vectors exerted upon that element by all water molecules (Brugé et al. 1994, 1996 a and b; Martorana et al. 1996 and 1997; Bulone et al. 1997). Direct solute-solute forces were not taken into account. Related errors (≤ 4 pN) and statistical significance were evaluated as in Flyvbjerg et al. (1989) and as in Brugé et al. (1996 a) with identical results. The total force and torque acting on all solutes were seen to be zero within the same accuracy.

To obtain hydration patterns, the simulation box was divided into cubic cells and the probability Π that the center of the oxygen atom of a solvent molecule fell within the cell was computed. The average probability Π_0 relative to the unperturbed solvent was similarly computed, and the ratio $\Pi/\Pi_0 = P$ was also computed. In this way, P was normalized to unity at large distances from solutes. The side length of the cells was 0.35 Å for two-solute simulations and 0.7 Å in all other cases. Using this space-occupancy probability P and the interaction potential and (whenever appropriate) the orientation of water molecules, contributions from each cell to SIFs acting on LJ solutes could be computed (Brugé et al. 1996 a) and summed up as an alternative way to obtain SIFs. In fact, this is equivalent to computing them as time averages of instantaneous (time-resolved) vectors. From time-resolved analysis of SIFs, normalized Fourier power spectra were obtained using the maximum entropy method (Press et al. 1986) with 40 poles. Impulses exerted by time-resolved SIFs were also computed (Brugé et al. 1994) for each time interval τ between two consecutive zeros of the given force (see Fig. 7b). Impulses of duration τ, $I_k(\tau)$ were added to obtain $I(\tau) = \sum_k I_k(\tau)$, that is the weighted distribution of impulse durations.

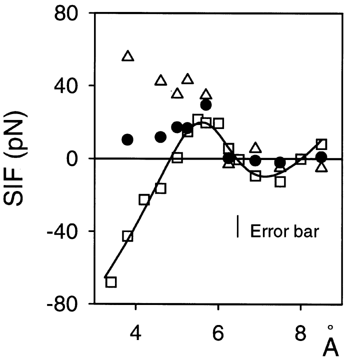

Fig. 1 Solvent induced force (SIF) between a pair of fixed simple spherical solutes, vs. their center-to-center distance. SIFs are computed as average values of instantaneous force exerted on each solute by all water molecules, along MD simulations of 800 ps or more. *Squares* and best-fitting polynomial *continuous line:* both solutes are modeled as uncharged particles, interacting with the solvent via a Lennard-Jones (LJ) apolar potential. The LJ parameters are the same as for the oxygen of water. *Circles:* one of the two solutes bears a unitary positive electronic charge. This is accounted for by adding the appropriate Coulomb term to the LJ interaction potential. *Triangles:* same, for a negative charge. Direct solute-solute forces not included. (Redrawn from Bulone et al. 1997)

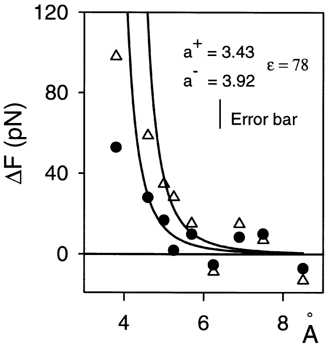

Fig. 2 Contributions to SIFs acting on two LJ solutes, due to the presence of an electric charge on one of them (see caption to Fig. 1). Data are calculated from those of Fig. 1 by subtracting SIFs acting on uncharged solutes from values obtained when one of the solutes is charged. *Circles:* positive charge. *Triangles:* negative charge. These contributions are compared to those calculated by classic electrostatics for the force acting between a point charge and an empty sphere in a continuum polarizable medium, with $\varepsilon = 78$. Best fitting of data points (*continuous lines*) are obtained using different effective radii for positive and negative charge (3.4 Å and 3.9 Å respectively). Even using these unphysical best fitting values, small distance data cannot be accounted for. The comparison shows the inadequacy of continuum models in accounting for hydrophobic-charged solute interactions on the microscopic scale (redrawn, from Bulone et al. 1997)

Results

a) SIFs within solute pairs

Results in Figs. 1–7 illustrate the main features of SIFs between a pair of LJ solutes, one of which bears in turn a zero, positive, or negative unitary electronic charge. Average SIF values as a function of solute-solute distance are given in Fig. 1. Force values for the case of uncharged LJ solutes (empty squares and continuous line in Fig. 1) exhibit the known feature of solvent-separated hydrophobic interactions, reflecting the particulate nature of the solvent (Pratt et al. 1980; Pangali et al. 1982; Ravishanker et al. 1982). The contact equilibrium configuration is expected to occur in the neighborhood of 3.5 Å. The solvent-separated configuration occurs at about 6.5 Å (Martorana et al. 1997). A positive or negative charge on one solute causes the occurrence of a repulsive contribution ΔF to

SIFs which fades out rapidly at distances larger than 6 Å. At shorter distances, ΔF is strongly charge-sign dependent, (always larger in the case of negative charge) and it overwhelms the hydrophobic contribution. In Fig. 2 ΔF values are compared with the force, calculated in terms of classical electrostatics, between a point charge and a spherical cavity in a continuum polarizable medium, with $\varepsilon = 78$ and optimal sphere radii determined by best fitting. At sufficiently large distance ($d \gtrsim 4.5$ Å), MD data follow a trend similar to that of the continuum model, under the (unphysical) choice of different cavity radii in the cases of positive and negative charge (3.4 Å and 3.9 Å, respectively). Even when using such ad hoc values, strong deviations from the continuum model description (stronger for negative charges) are observed at short distances.

The space-resolved origin of these features on the molecular scale is illustrated in Figs. 3–5. Figure 3 refers to the case of two uncharged LJ solutes, at the selected distances shown at the top. Hydration patterns are presented as 2D contour plots of space occupancy probability P (defined in Methods) along with space-resolved contributions to SIFs. It is of interest to see that relatively minor changes of hydration patterns correspond to large changes (and even to sign reversal) of SIFs. This is because the total SIF is a small difference between large opposite contributions. Actually, values of the total of either attractive or repulsive contributions fall in all cases in the range of 100–150 pN, to be compared with values of 20 pN at most for the resultant SIF. We note that the relatively small changes in hydration patterns illustrated in Fig. 3 would not be easily detected by X-rays or revealed by approximate, computer efficient methods. Hydration details highly significant for SIFs can therefore be easily missed, unless the molecular nature of the solvent is explicitly taken into account.

Figures 4 and 5 show the effects of the addition of a positive or negative charge on the solute to the right, for the particular center-to-center distance of 5 Å. In Fig. 4 2D contour plots of hydration and of space-resolved contributions to SIFs are shown, as in Fig. 3. In Fig. 5 we show a more pictorial 3D view of related iso-surfaces. This representation emphasizes differences of hydration of the solute to the left, due to the presence and sign of a charge on the solute to the right. We see that, as expected, the presence of a charge now causes significant changes in hydration patterns. At the 5 Å distance to which Figs. 4 and 5 refer, SIFs on the two solutes are: zero for the uncharged case (top); 17 pN (repulsive) for the positive charge case (center); 35 pN (repulsive) for the negative charge case (bottom). On each solute of a pair, SIFs are expected and found to the equal and opposite. Hydration and contributions to SIFs around the charged solute, however, illustrate how SIFs acting on it result from the balance of 10-fold larger contributions, compared to those acting on the uncharged solute. In fact, around charged solutes, the two opposite contributions to SIFs are in the range of 1000–1500 pN, while those around the uncharged partner are again in the range of 100–150 pN. Note that, as a consequence of the much larger values of contributions to SIFs acting on-

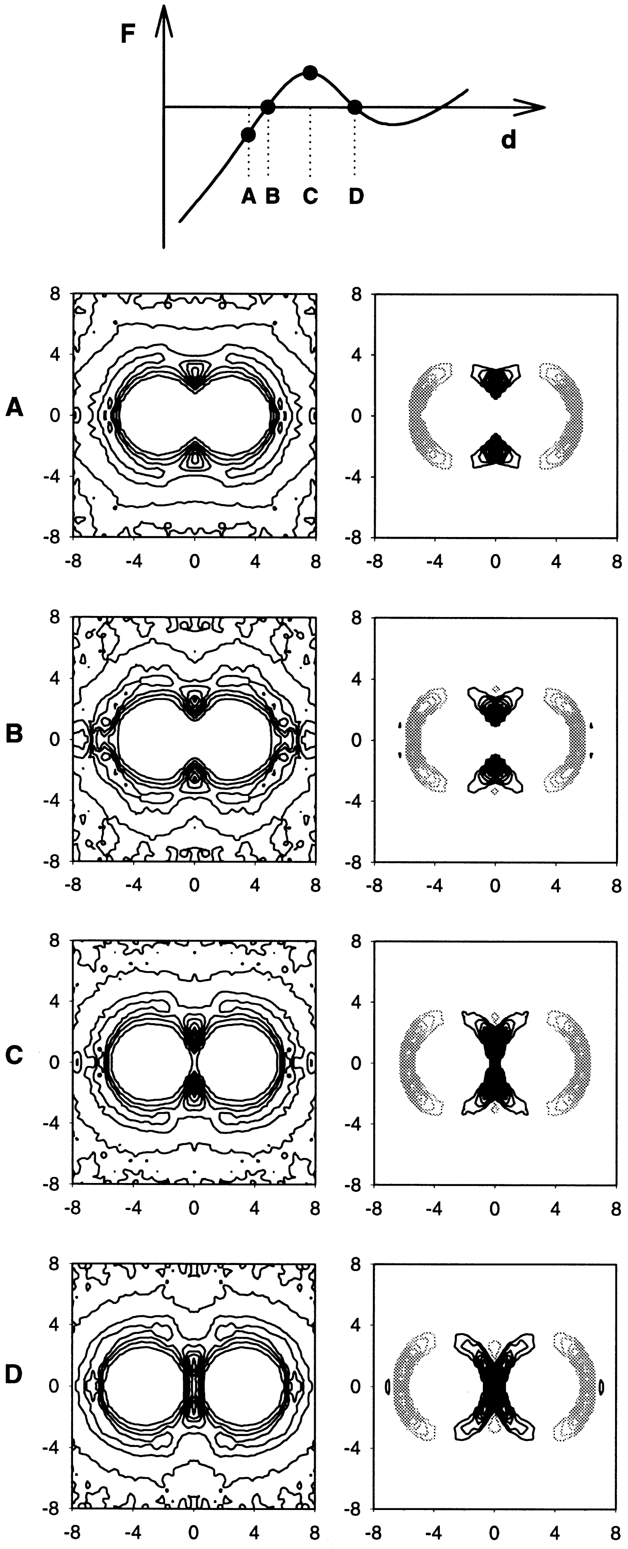

Fig. 3A–D At the top of this figure the continuous line of Fig. 1 is redrawn. For each of the four distances corresponding to points **A**, **B**, **C**, **D**, are shown on the *left* contour plots of space occupancy probability by water molecules, *P*, normalized so as to have $P \to 1$ at large distance from solutes. These contour plots are shown in increments of $\Delta P = 0.5$ up to maximum *P* values of the order of 10. Similar contour plots of SIF contributions are shown on the *right*, in increments of 0.025 pN, up to maximum contributions of 0.8 pN. *Continuous* and *dotted lines* refer to repulsive and attractive contributions respectively

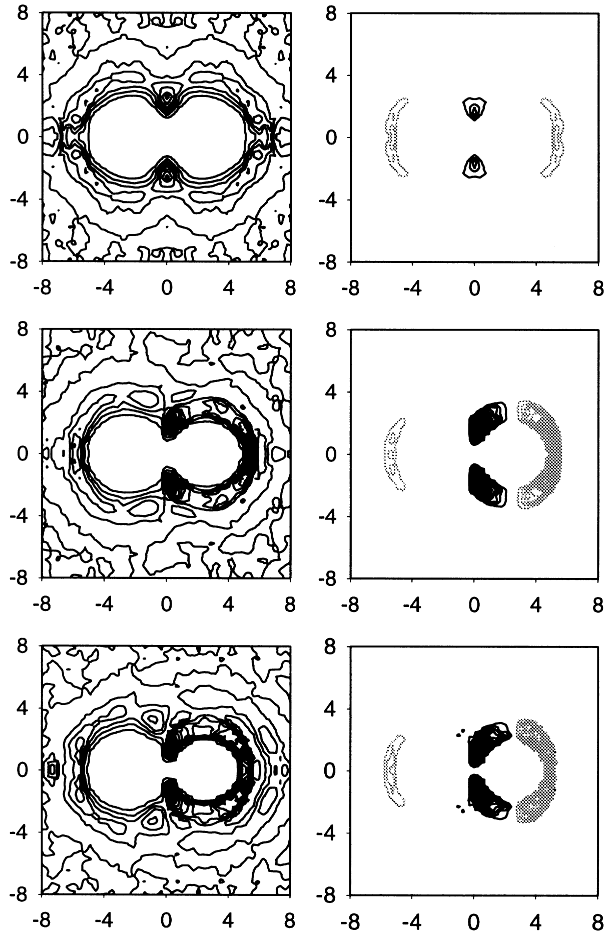

Fig. 4 Contour plots of space occupancy probability P (*left*) and space-resolved contributions to SIFs (*right*) as in Fig. 3. The solute to the right bears in turn a null (*top*), positive (*center*), negative (*bottom*) unitary electronic charge, while the solute on the left remains uncharged. Center-to-center distance of solutes is 5 Å, corresponding to case B in Fig. 3. Contour plots on the *left* are shown in increments of $\Delta P = 0.5$, up to maximum P values of the order of 15. Those on the *right* are in increments of 0.1 pN, up to maximum contributions of 4 pN. Continuous and dotted lines refer to repulsive and attractive contributions to SIFs as in Fig. 3

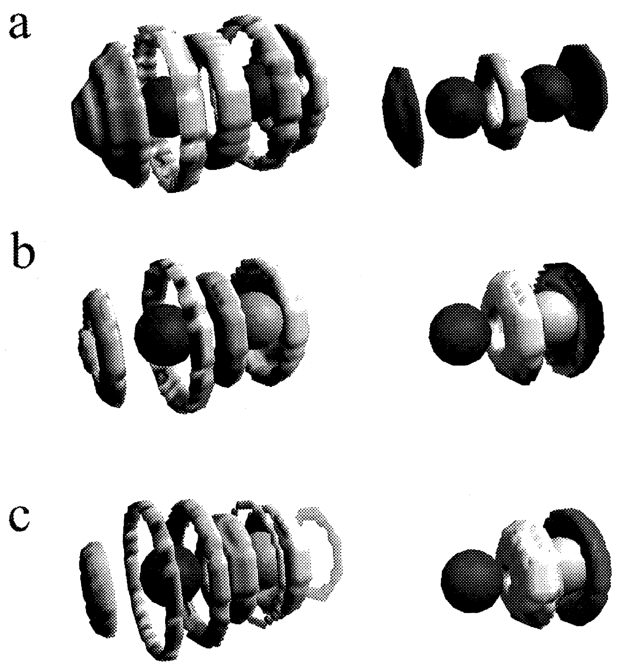

Fig. 5 a–c A 3D representation of the same data as in Fig. 4. Surfaces of equal probability (*left*) are at $P = 1.6$. Surfaces of equal SIF contributions (*right*) are at 0.25 pN in **a**, and 0.8 pN in **b** and **c**. Dark and light surfaces refer to attractive and repulsive contributions, respectively. Graphics from SciAn (Pepke and Lyons 1993)

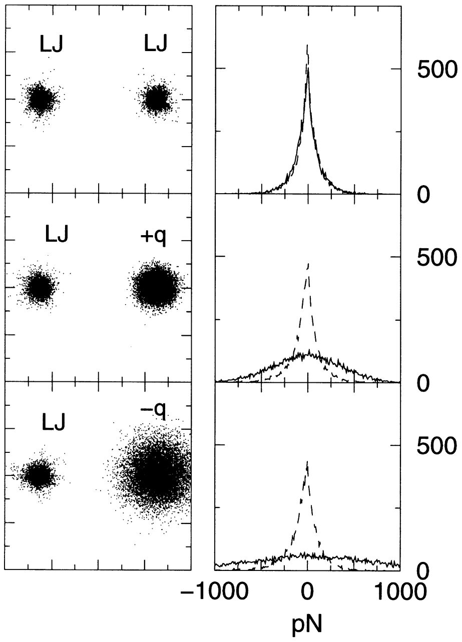

Fig. 6 *Left:* projection on the figure plane of the trajectory of the tip of the SIF vector, throughout the MD run. *Right:* statistical distributions of SIF components along the solute-solute direction (*dotted lines:* SIF on the solute to the left; *continuous lines:* SIF on the solute to the right). Solute types and configurations as in Figs. 4 and 5. Note how the thermodynamic SIF value results from the average of large instantaneous values of opposite sign. The range of instantaneous values is seen to be much larger in the case of charged solutes

charged solutes, scales used in Fig. 4 for contour plots to the right are significantly different from those used in Fig. 3.

While such space-resolved features provide information on the average distribution of the hydration water, time-resolved features provide complementary information on its dynamics. Information of this type is shown in Figs. 6 and 7 for the same solutes and configurations as in Figs. 4 and 5. Figure 6, left, was obtained by projecting on the plane of the figure the trajectories of the tip of the SIF vector acting on each solute of the three pairs. Force values and directions are seen to be much more widely fluctuating around their mean values for charged than for uncharged solutes, as quantitatively shown by statistical distributions in Fig. 6, right. This is because the force between water molecules and charged solutes is strongly sensitive to the orientation and related dynamics of water molecules.

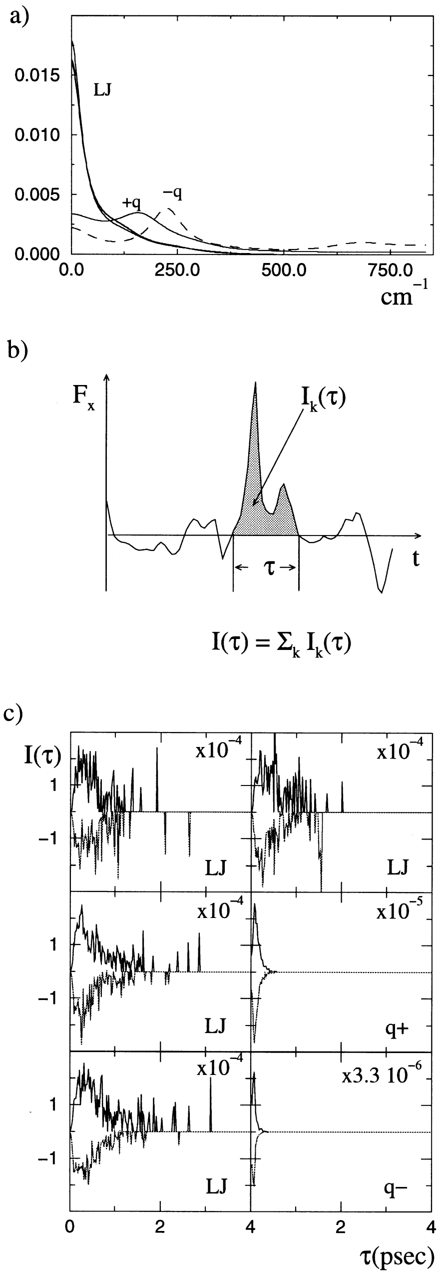

Fig. 7a–c Time-resolved features of SIFs acting on the same solute pairs as in Figs. 3, 4 and 5. **a** Normalized Fourier spectra. There are four essentially superposed continuous lines peaked on zero cm^{-1}. These lines refer to SIFs acting on uncharged solutes. They exhibit a broad, bareley resolved shoulder due to translational vibration of water. *Continuous* and *broken lines* with a pronounced peak in the 100–300 cm^{-1} range refer to SIFs acting on the positively and negatively charged solutes, respectively. The peak is due to an increase in frequency and intensity of the translational band, demonstrating more pronounced packing of hydration water, which is tighter in the case of negative charge. Note that the small band at about 700 cm^{-1}, caused by water librations, is visible in the case of a negative charge only, as expected. **b** Definition of single impulses $I_k(\tau)$ and their persistence time τ. **c** Weighed distributions of positive and negative impulses on each solute, vs. persistence time τ. Note that the duration of impulses acting on uncharged solutes extends up to 3 ps, suggesting a similar lower limit for permanence times of hydration water molecules. In the case of charged solutes, the impulse duration should instead be compared to the (much shorter) orientational time

Fourier transforms of SIFs in Fig. 7a agree with this view, as they show peaks in the spectra of SIFs acting on the charged solutes corresponding to translational vibrations (in the 200 cm^{-1} range) and librations (in the 700 cm^{-1} range) of water molecules (Eisenberg and Kauzmann 1969). When comparing the translational peak observed in the cases of negatively and positively charged solutes, we see that the first is significantly shifted towards higher frequency. In agreement with the hydration pattern data, this reveals that water molecules are more tightly bound to negative than to positive charges. Also, we see that the librational mode does not affect Fourier spectra in the case of the positive charged solute: this is because this mode only involves motions of the positive charges of water molecules, which are close to the solute when negatively charged, but far from it when positively charged. Notably, these features hardly appear in the spectrum of SIFs acting on LJ solutes (irrespective of the presence and sign of the charge on the partner solute). This fully agrees with the fact that such SIFs depend only on the center-to-center distance between solute and water molecules. In Fig. 7c we show the distribution of impulse durations, as defined in "Methods" and illustrated in Fig. 7b. We see that impulse durations in the case of uncharged LJ solutes go up to 3 psec or more, suggesting a similar lower limit for permanence times of hydration water molecules. In the case of a charged solute, the duration of impulses is instead comparable with the much shorter orientational time. This is seen to be shorter in the case of negative charge, so indicating that hydration water molecules are in this case trapped in steeper potential wells.

In summary, this first part of our results shows that: i) adding an electric charge to one LJ solute of a pair can change the attractive hydrophobic force within the pair into a strong repulsive force; ii) the origin of this strong effect lies in the similarly strong attraction between the charged solute and solvent molecules; iii) owing to the asymmetric distribution of electric charges on water molecules, the effect depends strongly on the sign of the charge; iv) time-resolved features of instantaneous SIFs provide direct information on the dynamics of hydration water, as perturbed by solutes.

The above effects, particularly the charge-sign dependence, are strictly related to the particulate nature of the solvent, which must be taken into explicit consideration for their full appreciation.

b) Effects of a third solute on pairwise hydrophobic interactions

Further simulations reveal strong effects of a third charged or uncharged solute on hydrophobic interactions between two solutes. Three solutes are now used, in the configuration shown in Fig. 8 (inset). Solute 3 bears, in turn, a null, positive or negative unit of electronic charge. In different runs the 1–2 distance varies from 3.5 to 8 Å, while the third solute is maintained in a fixed position. Average values of SIF between solutes 1 and 2 (i.e. of components

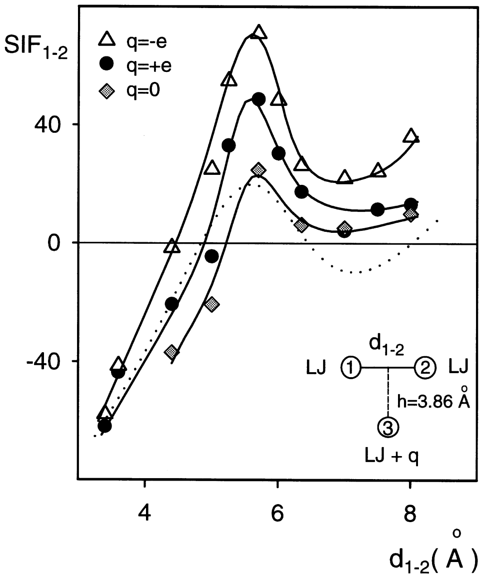

Fig. 8 Components along the 1–2 direction of average (thermodynamic) SIFs between hydrophobic solutes 1 and 2, in the presence of a third solute 3, in the configuration shown in the inset. Each point is obtained from a different set of runs at the corresponding 1–2 distance. For all runs h = 3.86 Å. Data points refer to uncharged (*diamonds*), positively charged (*circles*) and negatively charged (*triangles*) solute 3. Lines through data points are only a guide to the eye. The dotted line (redrawn from Fig. 1) is shown for comparison purposes, as it refers to the case when solute 3 is absent

along the 1–2 direction of SIF acting on solutes 1 and 2) are plotted in Fig. 8 as a function of the 1–2 distance. We see that at $d_{1-2} \leq 5.5$ Å the presence of a third uncharged LJ solute boosts the hydrophobic attraction between 1 and 2. The presence of a charge on solute 3 causes instead a strong and charge-sign dependent repulsive contribution, larger in the case of a negative charge. At short distances, this contribution weakens the hydrophobic attraction, while at larger distances it adds to the (already repulsive) force. Whether a solvent separated equilibrium configuration of solutes 1 and 2 still exists when solute 3 is charged is not clear from data presently available and more computing effort would be required to make it clear. Simple inspection of areas encompassed by curves in Fig. 8 shows, however that the negative part of the work necessary to bring solutes 1 and 2 in the contact configuration is remarkably increased by the presence of a charge on solute 3. In other words, the hydrophobic contribution of the free energy of the 1–2 pair is strongly reduced, and it might even become unfavorable. If so, the very concept of "hydrophobic bond" of two apolar groups would become questionable in the presence of a charged group (particularly so if negatively charged). Work to determine the full PMF curve is currently in progress. In Fig. 9, 3D iso-surface representations are used to show hydration patterns of the system and space resolved contributions to SIFs (1–2) for the three charge values (0, +q, –q) at a 1–2 distance of 5 Å. Time-resolved features of SIFs are similar to those for the solute pairs (Figs. 6 and 7) and are therefore not shown.

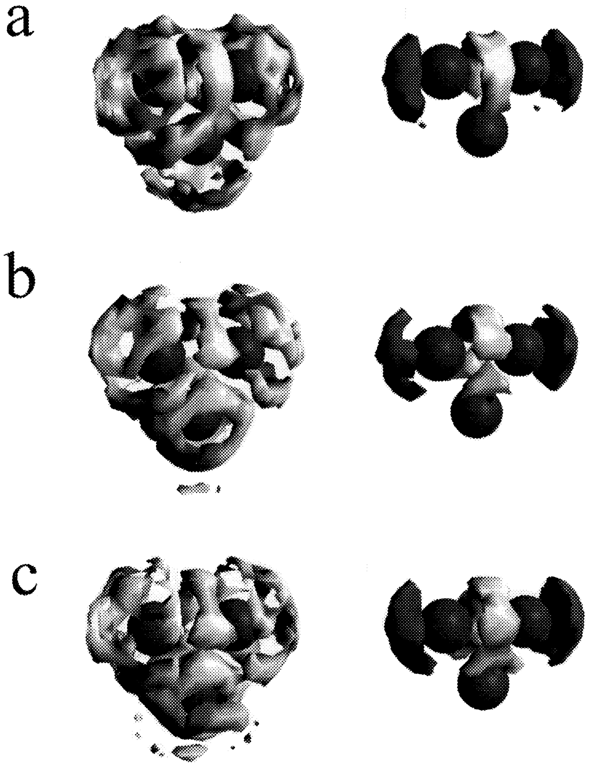

Fig. 9a–c A 3D representation of total hydration (*left*) and of space-resolved contributions (*right*) to the component along the 1–2 direction of average (thermodynamic) SIFs acting on solutes 1 and 2, in the configuration shown in Fig. 8, inset. This figure is analogous to Fig. 5 and refers to $d_{1-2} = 5$ Å. Solute 3 is uncharged in **a**, positively charged in **b**, negatively charged in **c**. Surfaces of equal probability are at $P = 1.6$. Surfaces of equal SIF contributions are at 0.8 pN. Dark and light surfaces refer to attractive and repulsive contributions, respectively. Graphics from SciAn (Pepke and Lyons 1993)

c) SIFs on a composite solute, containing apolar and charged elements

In Fig. 10–12 we show results concerning composite solutes, containing 5 or 6 elements in fixed planar configuration. In Fig. 10 all elements are uncharged LJ spheres (for a partial report of these data, see Martorana et al. 1997). The figure is organized in four pairs of panels, labeled a, b, c, d. The top panel of each pair shows SIFs on each element, computed additively (that is in the KSA) from data for the pairs in Fig. 1. The bottom panel of each pair shows true SIFs acting on each element, as obtained from ad hoc MD runs. In Fig. 10a, the arrangement is an array of equilateral triangles, at a nearest neighbor center-to-center distance of 4.6 Å. The pairwise SIF between nearest neighbours is seen from Fig. 1 to be 16 pN. At the largest distance (12.17 Å, between elements 3 and 4) pairwise SIF as well as direct LJ–LJ forces are zero. Large non-additivity effects cause a reinforcement of hydrophobic forces which drive the system towards hydrophobic collapse. In the next panels b, c and d, one of the elements is missing in turn. In case b, the missing element is number 1. Non-additiv-

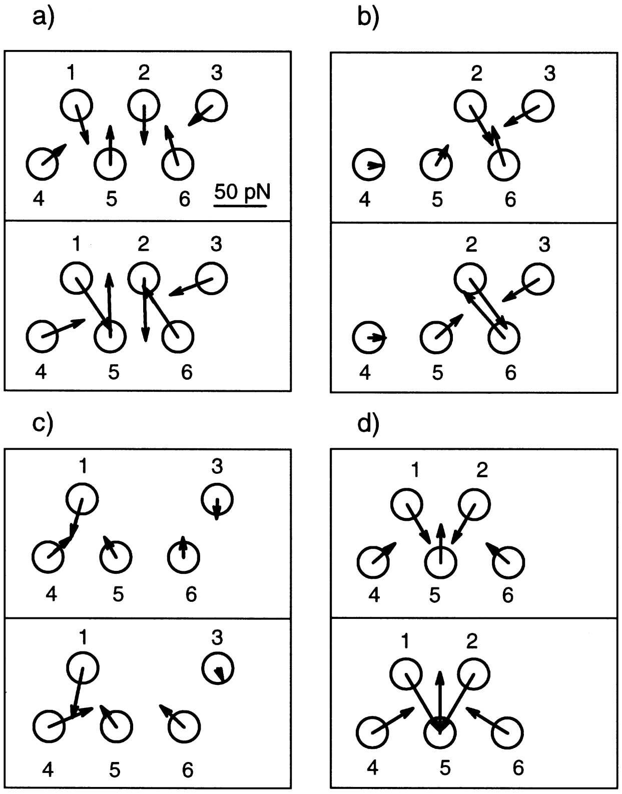

Fig. 10a–d Average (thermodynamic) SIFs acting on each element of a composite solute, made of 5 or 6 LJ elements, in the planar configuration shown. All nearest neighbor distances are 4.6 Å. The largest distance (between elements 3 and 4) is 12.17 Å. In each pair of panels, the one at the top shows the SIF vectors computed from pairwise data of Fig. 1, in the additivity hypothesis (corresponding to Kirkwood's superposition approximation). The *bottom panel* of each pair shows the "true" SIF vectors obtained from MD simulations. **a** 6-element composite solute. **b** Element 1 is missing. As a result, particles 2, 5 and 6 are more strongly attracting each other. **c** Element 2 is missing. Particle 3 is now driven away from other solute elements and towards the solvent, despite its hydrophobic (apolar) character. **d** Element 3 is missing. Compared with case **a**, SIF acting on particle 4 is stengthened by a transverse 10 pN contribution. Note that the 3–4 distance is such as to rule out any direct or solvent induced pairwise interaction. Differently stated, no force at all would act on 3 and 4 in the absence of other solute elements. The observed change reveals a long-distance interaction occurring by way of a relay action through the interposed solutes, caused by the collective character of hydration

ity enhances the drive on particles 2, 3, 5 and 6 towards hydrophobic collapse, while it affects weakly SIF on particle 4. In case c, particle 2 is missing. Particle 3 is now driven by SIF towards the solvent, despite its hydrophobic character. In case d, the missing element is number 3. We see that this causes a considerable change of the SIF vector on element 4 (a transverse 10 pN contribution) which would not occur in the case of pairwise additivity, that is in KSA. In orther words, an interaction between the distant elements 3 and 4 occurs at a distance sufficiently large to rule out pairwise interactions of any kind. This interaction is made possible by the presence of the intervening particles 1, 2, 5 and 6. As we have already recalled, the or-

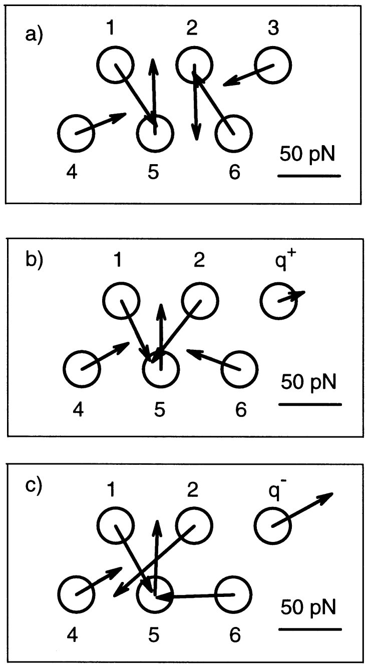

Fig. 11a–c Average (thermodynamic) SIFs acting on elements of the same composite solute as in Fig. 10a. *Top:* redrawn for comparison purposes from Fig. 10a (all elements are uncharged). *Centre:* element 3 bears a positive charge. *Bottom:* element 3 bears a negative charge. The effect of charges (stronger for the negative case) is to enhance the drive towards hydrophobic collapse of elements 1, 2, 4, 5, 6

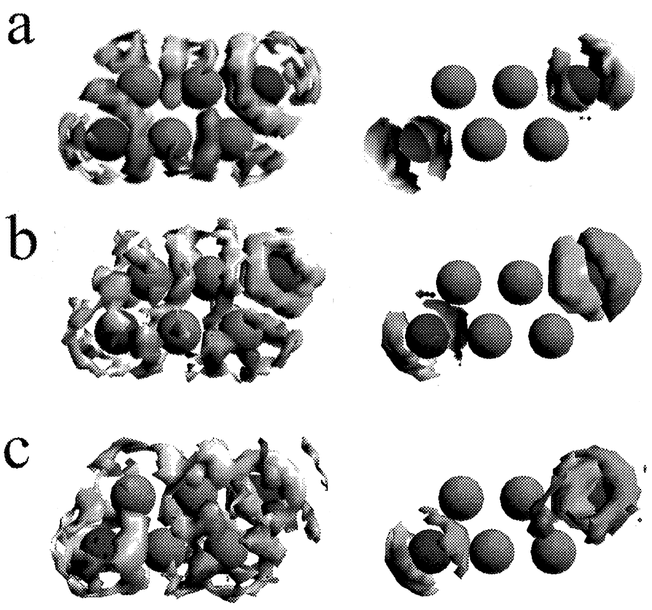

Fig. 12a–c A 3D representation (analogous to Fig. 5 and Fig. 9) of hydration (*left*) and of space resolved contributions to average (thermodynamic) SIFs acting on elements 3 and 4 (*right*). The composite solute is as in Fig. 11. *Top:* all elements are uncharged. *Centre:* element 3 bears a positive charge. *Bottom:* element 3 bears a negative charge. Surfaces of equal probability are at $P=1.6$ and at 0.5 pN, respectively

igin of SIFs and of their non-additivity lies in the overlap of hydration of individual elements and in the consequent appearance of δG_{SW} contributions of different orders in the total free energy of hydration (Eq. (1)). Therefore, the case in Fig. 10d shows that a relay action transmitted self-consistently along overlapping hydration regions of all solute elements causes high-order δG_{SW} contributions and a consequent significant extension of the range of solvent induced interactions. This relay action is closely related to the context-dependence of individual SIFs, responsible for reversing in some cases (as in Fig. 10c, but see also the following section) individual SIFs. The resulting sign reversal would appear counterintuitive on the grounds of expectations based on widely accepted notions such as "hydrophobic" and "hydrophilic". Its occurrence, however, is not surprising in terms of non-zero high-order δG_{SW} terms.

These conclusions are endorsed and made clearer by Fig. 11 where we show SIFs acting on each element of the same composite solute when a positive or negative charge is added to element 3. We see that the charged element is driven away from the apolar part by a SIF which is larger in the case of a negative charge. This agrees with data concerning two-particle systems in Figs. 1 and 2, and three-particle systems, in Fig. 8. However, at variance with the case of Fig. 8, we see that the group of apolar elements is more strongly driven towards hydrophobic collapse. In the space resolved analysis visualized in Fig. 12, hydration patterns and space resolved contributions to SIFs on elements 3 and 4 are compared for the three cases of a null, positive and negative charge on element 3. Here again we see that relatively modest changes of hydration cause large changes of SIFs. This is because, as already noted, and as visible from the right part of the figure, the net value of SIFs is a small difference of large opposite contributions.

d) SIFs on a protein, at single residue resolution

SIFs at single residue and at single side chain resolutions have been obtained by MD simulation of bovine pancreatic trypsin inhibitor (BPTI) in water. Results, already reported elsewhere along with methods (Martorana et al. 1996 and submitted), have shown marked effects of long-range correlation and context dependence. These effects were seen to be caused by the fact that the hydration pattern is not uniquely determined by the specific character (charged, polar, apolar) of each side chain, but rather collectively determined by the whole protein. The context-dependence is such as to cause correlations of time-dependent SIFs at distances as large as the entire protein (up to 27 Å). Also, in a few significant cases, it causes a sign-reversal of SIFs, with respect to expectations based on the specific character of the side chains, much as observed in the model system of Fig. 10c. As an example of this apparently counterintuitive context dependence, we show in Fig. 13 the SIF vector acting on similar groups (three identical hydrophobic prolines) in different positions. From top to bottom, we observe a SIF directed towards the center of the protein (as expected for proline), along the protein surface, or towards

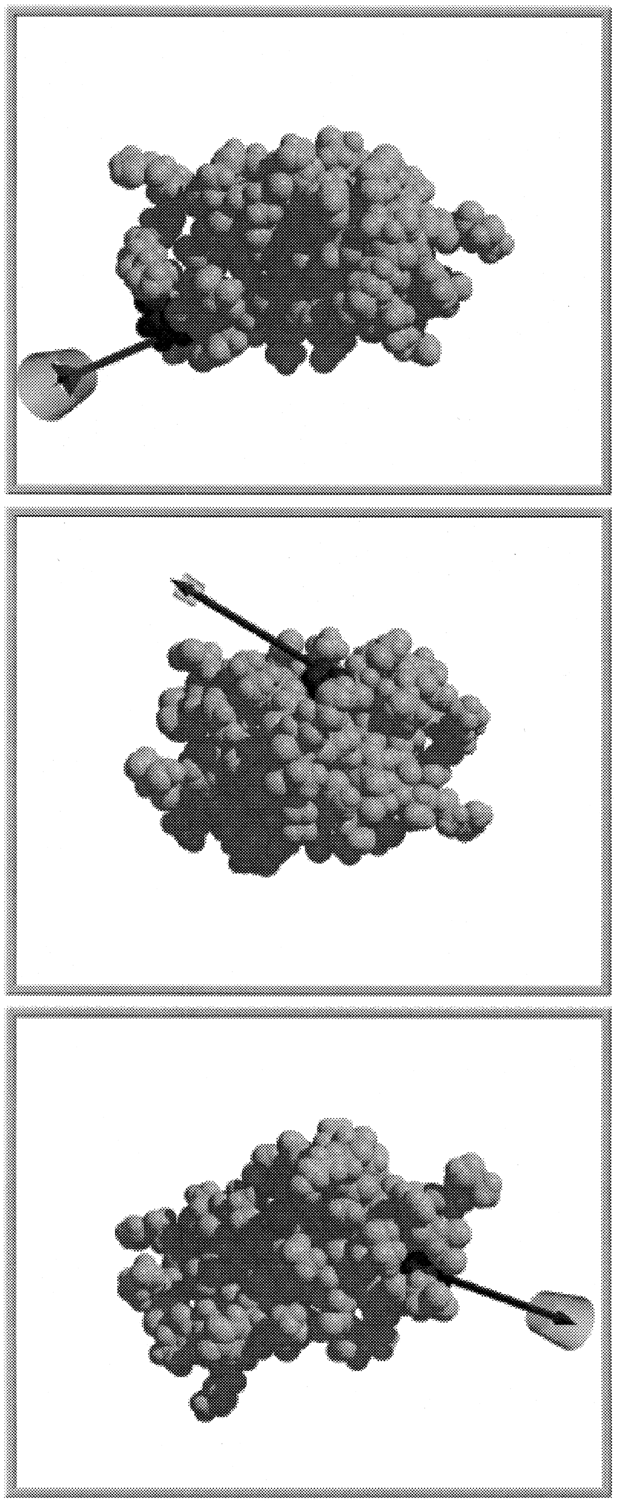

Fig. 13 Average (thermodynamic) SIFs acting on (hydrophobic) sidechains of prolines occupying three different sites of a protein (BPTI), *Top:* SIF driving Pro 13 away from the solvent, in agreement with predictions based on the hydrophobic character of this residue. *Centre:* SIF on Pro 8, essentially tangential to the protein surface, not quite in agreement with predictions based on the hydrophobicity of this residue. *Bottom:* SIF driving Pro 2 towards the solvent, at odds with predictions based on hydrophobicity of this residue. These three cases show how the spatial context can riverse the hydrophobic force as if the given apolar element were hydrophilic. (From the MD trajectory of Martorana et al. 1996)

the solvent (contrary to expectations and as would infact be expected for a hydrophilic group). All together, these results show very convincingly the context-dependence of solute-solvent interactions. They also show how the collective character of PMF and hydration in a complex system such as a protein can cause, through a relay action involving interposed solute, long-distance correlation and interactions.

In closing this section it is in order to ask to what degree of confidence can we take the collective character of hydration and SIFs and their consequent properties evidenced here. We note that these features cannot be artifacts due to a particular choice of water-water potentials. In fact, they emerge from the data of Brugé et al. (1996) obtained with the use of ST2, from results in sections a) b) and c) obtained using TIP4P, from results in section d) and in Martorana et al. (1996) obtained with MCY. Use of flexible/polarizable potentials was not attempted, but it can be expected to emphasize non-linearity, modulability and long-range extension of interactions.

Discussion and conclusions

The exquisite specificity of protein functional interactions calls for a complete understanding at atomic resolution of all relevant interaction mechanisms. These include solvent-induced interactions and SIFs because their work, as already recalled, can have a central role in protein function and folding. Viewing SIFs within clustered solute elements such as protein residues at single element/residue resolution is therefore instrumental to our understanding of how proteins fold and work. For this purpose, MD simulations with explicit solvent as used in the present work are necessary. This is because truncations or averagings of high order interactions used in theoretical treatments such as of semidilute solutions (e.g. virial expansion) or in highly efficient simulations will necessarily blur all fine details. So, we do not expect such approximate treatments to be always adequate on the microscopic scale and at the concentrations which are significant in the case of specific biological interactions. They are nevertheless adequate whenever fine details are not required. In fact, a number of experiments (San Biagio and Palma 1991; Sciortino et al. 1993; Bulone et al. 1993; San Biagio et al. 1996a and b) and theoretical models (de Gennes 1979; Dill et al. 1995) show that mean-field approaches can provide adequate descriptions of biologically important gross grained events in complex systems. This is because the scale length of phenomena involved and the large number of interactions present in such cases allows *averaging* (not *ignoring*) non linear contributions. An approach in terms of average non additive effects has proved adequate down to the scale of some 1,000 Å^2, for a determination in quantitative terms of the work of SIFs in the T–R transition of hemoglobin (Bulone et al. 1991, 1992, 1993; Palma et al. 1994).

Results in this work illustrate many novel or recently elicited features of SIFs on the microscopic scale. These are: strong non additivity; extension of the range of action of SIFs due to interposed solute elements; modulability of hydrophobic forces by electric charges and its dependence on charge sign. All these features are jointly due to the strong many body character of hydration and PMF. Thus, substitution of PMF for plain solute-solute potential does not simply amount to the addition of a smooth contribution. Instead, the particulate nature of the solvent and the great variety of its configurations endow SIFs and PMF with a context-dependent character, not infrequently causing *locally* counterintuitive or unconventional interactions. This agrees with and generalizes Anfinsen's prescient "thermodynamics hypothesis", stating that the functional folded form of a protein is one in which the free energy *of the whole system* is lowest (Anfinsen 1973). In this view, optimization of the total free energy may well occur at the expense of some unconventional *local* feature. That is, *local* optimization of free energy in terms of the individual character (hydrophobic/philic, charged) of each single residue taken as in isolation does not necessarily coincide with *global* optimization. It follows that notions such as hydrophobic or hydrophilic interactions are certainly useful but they should not be taken too strictly. Our studies on solutes of diverse size and complexity show how the strongly collective character extends in fact across a whole protein, giving hydration and related SIFs acting on local sites a very specific dependence upon the entire context of solute elements around that site. In Figs. 10, c and 13 this high specificity of SIFs and the fact that they are not simply amenable to additive effects of individual groups is illustrated for diverse scale lengths. This leads us to conclude that inter- and intra-biomolecular recognition can, not infrequently, occur along paths contradicting conventional expectations based on local properties only. Counter-intuitive steps of this type must be included in our predictive approaches to issues such as protein functional recognition and interaction, folding (including chaperoning), induced pathological conformational changes and coagulation. Given the size of these effects and of SIFs themselves, there is at present no reason for ruling out a central (if not necessarily very frequent) role of such counter-intuitive steps.

The significance of the present results can be seen to extend beyong the strong evidence for a need of diversified predictive approaches to inter- and intra-biomolecular recognition and interaction. For example, let us consider the frequently observed, small and yet biologically important non additivities of point mutation effects in proteins (Green and Shortle 1993; LiCata and Ackers 1995). Since protein function involves conformational changes or intermolecular encounters, it implies changes of total protein hydration and related free energy changes, ΔG_{SW}. This free energy change associated with the protein function will contain contributions from all δG_{SW} terms of Eq. (1). Mutations at even distant sites are therefore capable of altering, non additively *via* related δG_{SW} terms, the ΔG_{SW} value and thus the function. Such long-distance interaction *via* propagation of hydration perturbations is shown in Figs. 10, 11 and 12. From these figures it can be inferred that the strong many body character of hydration, PMF and

G_{SW} provides a general molecular mechanism acting through "some global elements of the protein", as sought by LiCata and Ackers (1995). The "global elements" are the statistically populated configuration and dynamical states of the two complex interacting systems: protein $+nH_2O$ (see Appendix and also Bulone et al. 1993; Palma et al. 1993 and 1994).

Another significant example refers to results in Figs. 8, 9 and 11. These figures show the need for extending and diversifying conventional, simplified approaches to the causal understanding of ubiquitous functional concomitance of changes of protein charges and conformation. A paradigmatic case of such interdependence is of course the relation of phosphorylation to mechanical work. Another example illustrating the role that features of SIFs presented here can have, is the crucial specificity of modular binding domains for pTyr in signal transduction proteins. In this case, binding specificity is modulable over a remarkable span of three orders of magnitude, by just changing one residue at a given site (Cohen et al. 1995).

In conclusion, this work illustrates properties of non-additivity, modulability, context-dependence and long-range action of solvent-induced forces and interaction, and of their well known subset, hydrophobic interactions. The ubiquitous presence of SIFs, the size of the work that they can perform (to be compared, for example, with free energy values stabilizing protein conformation), and their so far unrecognized character of high specificity and equally specific modulability by electric charges, illustrate the need for taking them into account in understanding protein function in terms of basic physical laws.

Acknowledgements We gratefully acknowledge discussions and long-term collaboration with A. Emanuele, S. L. Fornili and M. Manno, and help from D. Giacomazza. Partial support from the Italian "Ministero per l'Università e la Ricerca Scientifica e Tecnologica" and from the Sicilian "Comitato Regionale Ricerche Nucleari e Struttura della Materia" is also acknowledged.

Appendix

Insight into the physical origin of SIFs and their unusual properties can be provided if unambiguous definition is given of statistically populated configurations of the solvent (that is of its "structure") and of their relation to G_{SW}. As first recognized by Fisher (1964), structural characterization of liquids should be done in terms of vibration-averaged configurations (V-structure in the terminology introduced by Eisenberg and Kauzmann 1969). This is also required for an appropriate definition of the H-bond (Naberukhin 1981 and 1984) and for numerical studies concerning the H-bond network (Hirata and Rossky 1981; Belch et al. 1981; Zichi and Rossky 1985). Related approaches focus on frozen structures (Malenkov 1984; Medvedev et al. 1987; Malenkov et al. 1987 and 1989) or inherent structures (Weber and Stillinger 1984; Stillinger 1988). The latter has been carried out to provide an explicit expression for free energy (Stillinger 1988), particularly useful in the present case.

Water configuration can be viewed in ordinary geometric space as well as in the more abstract multidimensional configuration space. Let us first consider the total potential energy of a system of N molecules of bulk water. This is a function of nN configurational variables (n is the number of degrees of freedom) and it can be thought of as a "landscape" in the related multidimensional space. In such space, the set of water configurations corresponding to (relative) minimal of potential energy is referred to as "inherent structures" (Weber and Stillinger 1984; Stillinger 1988). The whole configurational space can be partitioned into "basins" surrounding each minimum. The dynamics of the system are described by the motion of the representative point of the system within basins and hopping among them. Hopping can be identified as structural relaxation events (Tanaka and Ohmine 1989; Ohmine and Tanaka 1990). The time scale of the latter is determined by the multiplicity of paths, distances and barriers among basins. Under given thermodynamics conditions (say, N, V, T) only a small subset of basins is populated with overwhelming probability. Their depths φ, that is the potential energies at their minimal, fall within a very narrow range of values (essentially the same for all of them). Their multiplicity is a function of φ and N, and can be expressed as $e^{\sigma(\varphi)N}$. Accordingly, the free energy of the system can be written as

$$G(N,V,T) \simeq \varphi - Nk_B T\,\sigma(\varphi) + G_{vibr}(\varphi, T) \qquad [A1]$$

where G_{vibr} is a free energy contribution of vibrational motions within basins (Stillinger, 1988). Statistically populated basins identify statistically relevant solvent configurations in ordinary geometric space. Hopping among them accounts for the homogeneous density of bulk water.

As a consequence of excluded volume and interaction potential, a solute alters the landscape of potential energy in the solvent configurational space. More precisely, it alters the set of populated basins (and related configurations in the geometric space), their depth, multiplicity and dynamical contributions. These alterations involve the configurations of solvent molecules defining "solvation" ("hydration" in the case of an aqueous solvent). The consequent free energy change is the hydration free energy, G_{SW}. In most cases, the multiplicity of available basins is expected to be reduced by the presence of solutes, e.g. as a consequence of excluded volume. This causes the known longer residence time, altered enthalpy and dynamics, and lower entropy of hydration water. The effect of a solute on the energy landscape already perturbed by other solutes is of course not the same as in absence of other solutes and it depends upon solute-solute distances and mutual configurations. More precisely, and with reference to Eqs. [1] and [2] in the Introduction:

$$G_{SW}(1,2\ldots, n) = [\Delta\varphi - Nk_B T\,\Delta\sigma(\varphi) + \Delta G_{vibr}]_{(1,2\ldots,n)} \qquad [A2]$$

and the SIF acting on the h-th solute is

$$SIF_h = -\mathrm{grad}_h[\Delta\varphi - Nk_B T\,\Delta\sigma(\varphi) + \Delta G_{vibr}]_{(1,2\ldots,n)} \qquad [A3]$$

These expressions for G_{SW} and SIFs help in visualizing the concept of SIFs as the solvent contribution to the thermodynamic drive towards solute configurations minimizing the overall solute-solvent free energy. We see from Eqs. (A1)–(A3) that such solute configurations correspond to a potential energy landscape which is more favorable in term of depth and multiplicity of the statistically populated subset of basin.

It is of interest to compare expressions of SIFs in terms of inherent structures (Eqs. (A1)–(A3)) and of PMF. Let us consider a system of n solutes in fixed positions $(r_1 \ldots r_n)$ and (N–n) solvent particles. Solute-solute PMF, $\Psi(r_1 \ldots r_n)$ is implicitely defined by the equation:

$$g^{(n)}(r_1 \ldots r_n) = \exp\left[-(k_B T)^{-1}\, \Psi(r_1 \ldots r_n)\right] \qquad [A4]$$

that is

$$\Psi = -k_B T \, \ln g^{(n)}(r_1 \ldots r_n)$$

where $g^{(n)}(r_1 \ldots r_n)$ is the n-particle solute-solute correlation function. Ψ is the sum of a direct solute-solute potential, that we may disregard for the present purposes (as independent of solvent configurations), and of a solvent-induced contribution. Its knowledge requires an expression of $g^{(n)}$ which in turn leads to a hierarchy of equations linking $g^{(n)}$ to solute-solvent correlation functions of increasing order (see e.g. Hill 1956; Croxton 1975). Handling such a set of equations requires some kind of truncation of closure. In this statistical treatment the visualization of hydration and related free energy and forces remains implicit. In a complementary way, inherent structures offer a unified, more explicit view of solute-solvent interactions, of hydration and SIFs, in terms of the global alteration of the configurational energy landscape.

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