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pH dependence of the hydrogen exchange in the SH3 domain of α-spectrin

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Abstract **Using nuclear magnetic resonance we have measured the hydrogen exchange (HX) in the Src homology region 3 (SH3) domain of α-spectrin as a function of pH*. At very acidic pH* values the exchange of most residues appears to occur via global unfolding, although several residues show abnormally large Gibbs energies of exchange, suggesting the presence of some residual structure in the unfolded state. At higher pH* HX occurs mainly via local or partial unfoldings. We have been able to characterize the coupling between the electrostatic interactions in this domain and the conformational fluctuations occurring under native conditions by analyzing the dependence upon pH* of the Gibbs energy of exchange. The SH3 domain seems to be composed of a central core, which requires large structural disruptions to become exposed to the solvent, surrounded by smaller subdomains, which fluctuate independently.**

Key words: Amide–hydrogen exchange; Protein folding; Calorimetry; Src homology region 3 domain; Nuclear magnetic resonance

1. Introduction

The measurement by nuclear magnetic resonance (NMR) of hydrogen exchange (HX) under native conditions provides us with residue-specific, energetic information about the conformational states of proteins infinitesimally populated in the high-energy region of the folding landscape (see [1] for a recent review). The native state HX method relies upon the use of progressively destabilizing conditions to promote either partial or global unfolding reactions selectively in such a way that they dominate the exchange. In some proteins partially folded forms, often resembling the kinetic folding intermediates, have been identified and characterized [2–7]. Under high stability conditions, on the other hand, HX in many proteins appears to be dominated by small, localized fluctuations, which obscure the larger unfoldings which might be informative of folding intermediates [8]. These fluctuations seem to be responsible for the great heterogeneity observed in the pattern of HX protection factors in most proteins. To date the nature of these local structural motions remains obscure, although HX patterns under native conditions have been modeled reasonably well for a number of proteins by taking them to be statistical ensembles of partially folded states [9–12].

The α-spectrin Src homology region 3 (SH3) domain has long been considered a good model for two-state folding, both in equilibrium [13] and kinetic studies [13–15]. The folding reaction of this small domain occurs via a well-defined transition state, which is structured around the distal loop β-hairpin and the short 3_{10} helix [16]. On the other hand, we have found recently that under native conditions (mildly acidic pH and room temperature) the HX of this domain occurs mainly via local or partial unfolding reactions and therefore the scale of co-operative interactions in this domain is regional rather than global [12]. To explore further the nature of the conformational changes that proteins undergo under native conditions we have extended the HX study of the α-spectrin SH3 domain by increasing the pH range of the measurements from conditions of high stability (about 20 kJ mol^{-1}) to those where stability is marginal (about 4 kJ mol^{-1}). Our results provide an interesting picture of the conformational ensemble sampled by the polypeptide chain.

2. Materials and methods

The SH3 domain of wild-type chicken α-spectrin was isolated as described elsewhere [12]. Protein aliquots were extensively dialyzed against pure water and lyophilized.

Hydrogen–deuterium exchange was initiated as described elsewhere [12] by rapidly dissolving the lyophilized protein in deuterated buffer (20 mM d_5-glycine for pH* values of between 1.5 and 3.5 or 20 mM sodium d_3-acetate for pH* 4.0). The final protein concentration was approximately 4.5 mM. All exchange experiments were made at 24.7°C on a Brüker AMX-500 spectrometer. The probe temperature was determined before each experiment using a standard of 80% ethylene glycol, 20% d_6-dimethyl sulfoxide. A set of 20–30 two-dimensional, phase-sensitive COSY spectra were acquired as described elsewhere [12]. The spectra were processed using NMRpipe [17] and analyzed with NMRview [18]. The decay in intensity of each NH-C_αH cross peak with exchange was obtained from the two-dimensional spectra as described elsewhere [12]. A single exponential decay function was fitted to the decays to determine the exchange-rate constants, k_{ex}, for each amide proton. The intrinsic exchange-rate constants, k_{int}, at the different pH* values assayed were calculated using the exchange data of Bai et al. [19] for model peptides. The errors in k_{ex} were calculated from an analysis of the variance–covariance matrix of the fittings at a 95% confidence interval, using the fitting option of the Origin 5.0 program (Microcal Inc.). No errors were considered in k_{int}.

Under our experimental conditions the intrinsic exchange-rate constants for all amide protons were at least one order of magnitude lower than the refolding-rate constant of α-spectrin SH3 [13].

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Abbreviations: SH3, Src homology region 3; HX, hydrogen–deuterium amide exchange; pH*, pH meter reading for deuterium oxide solutions uncorrected from isotopic effects

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Accordingly, an EX2 mechanism for the amide–hydrogen exchange could be assumed. Under EX2 conditions the equilibrium constant, K_{op}, for the opening process, which renders the amide hydrogen of any particular residue exchange-competent, is calculated as $K_{op} = k_{ex}/k_{int}$. The apparent Gibbs energy difference between the open and closed states, referred to in this work as 'Gibbs energy of exchange', is given by:

$$\Delta G_{ex} = -RT \ln K_{op} \quad (1)$$

3. Results and discussion

We used two-dimensional NMR to measure the hydrogen–deuterium exchange in the SH3 domain of chicken α-spectrin at 25°C and six different pH* values: 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0. At pH* 1.0 all the residues exchanged within the dead time of the experiment and at pH* values of more than 4.0 the protein was not soluble enough for us to make the NMR measurements. At pH* 1.5 the HX could be measured for only 23 out of 59 possible amide hydrogens due to the fast intrinsic exchange rate at this pH*, whilst at other pH* values the number of amide hydrogens observable was between 35 and 39. The apparent Gibbs energy of exchange, ΔG_{ex}, for each residue at the different pH* values is plotted in Fig. 1. We have reported elsewhere the Gibbs energy change for global unfolding at each pH*, ΔG_{unf}, under identical conditions [12]. These values are also represented in Fig. 1 for comparison's sake. The uncertainties in ΔG_{unf} were estimated as ± 0.5 kJ mol^{-1}.

Surprisingly, both at pH* 1.5 and pH* 2.0 the ΔG_{ex} values of a few residues were significantly higher than the ΔG_{unf} values. This effect, sometimes referred to as 'superprotection' in the literature, has been interpreted as showing evidence for the deviation of the exchange kinetics from the EX2 limit [20,21]. This seems unlikely here since under our experimental

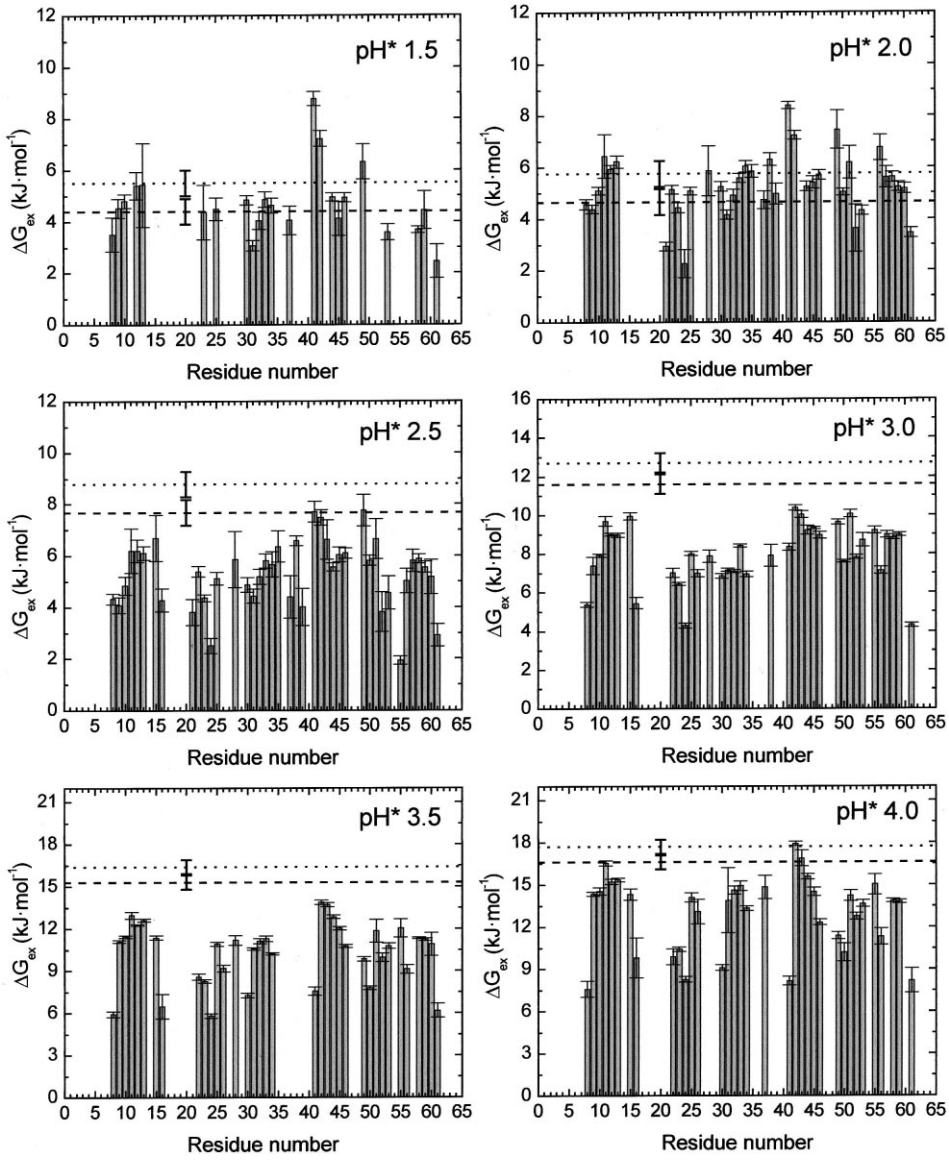

Fig. 1. The pH dependence of the HX in SH3 as detected by NMR. pH* values are indicated in each panel. Solid bars represent the Gibbs energies of exchange, ΔG_{ex}, for each residue. The estimated errors of these quantities (see Section 2) are represented above each bar. The global unfolding Gibbs energy at each pH* value is indicated by horizontal lines, both uncorrected (dashed lines) and corrected (dotted lines) for the effect of proline isomerization. Thick error bars indicate an uncertainty of ± 0.5 kJ mol^{-1}.

conditions the folding rates of α-spectrin SH3 have been shown to be very fast compared to the intrinsic exchange rates [13,15,16].

Slow proline isomerization may also account for discrepancies between ΔG_{ex} and the ΔG_{unf} value measured in an equilibrium unfolding experiment [20]. This has been put down to the fact that the isomerization equilibrium cannot relax during the fast structural fluctuations that lead to exchange. For two prolines in *trans* conformation in the folded state, the relevant ΔG_{unf} for HX studies needs to be corrected by adding 1.1 kJ mol^{-1}. Using this correction, the significantly superprotected residues are confined to Trp41 and Trp42 at pH* 1.5 plus Arg49 and Ala56 at pH* 2.0.

Some studies have explained superprotection as being due to the presence of residual structure impairing the exchange of some residues in the unfolded state [22,23]. This would lead to an overestimation of the intrinsic exchange-rate constant, k_{int}, relevant to the unfolded chain and to an abnormally high ΔG_{ex} for these residues. The presence of two tryptophans among the superprotected residues suggests some propensity of the chain to form a hydrophobic cluster at acidic pH. Very recently Kortemme et al. [24] used high-resolution NMR at pH 2.2 to demonstrate the tendency of the unfolded state of SH3 to sample the native topology in the central triple-stranded β-sheet. This is consistent with the unexpected ΔG_{ex} values observed here for some residues in the same region. Thus the unusual ΔG_{ex} values for these few residues appear to be meaningful and provide a glimpse of the conformational propensity of the unfolded chain at acidic pH.

Fig. 2 shows the change with pH* in the distribution of the ΔG_{ex} values. At low pH* values, where the stability of the domain is marginal, the distributions are narrow and converge with the Gibbs energy of global unfolding, ΔG_{unf}, except for the superprotected residues, which appear as a small shoulder at higher ΔG values. This is consistent with the general observation in proteins that as destabilizing conditions are approached, by decreasing pH or by increasing the pressure, temperature or the concentration of the denaturants for instance, large unfoldings play a progressively more important role in the exchange [1,2].

At higher pH* values ΔG_{ex} increases for most of the residues, which is in accordance with the rise in stability observed for the SH3 domain. ΔG_{unf} increases faster than ΔG_{ex} with a rise in pH*, however, and above pH* 2.5 most residues have lower ΔG_{ex} than ΔG_{unf} values. The distribution of ΔG_{ex} values is relatively broad at the highest pH* assayed and consists mainly of two separate peaks. This suggests that local or partial unfolding reactions with lower Gibbs energy than that of global unfolding tend to govern the HX of SH3 under these conditions [12].

The thermodynamic reason for the pH dependence of protein stability is the coupling between the folding of the protein and a concomitant change in the apparent pK_a of its ionizable groups. We have reported in a previous paper that the pH dependence of ΔG_{unf} in the SH3 domain of α-spectrin can be explained satisfactorily by considering that several of the protein's carboxylic groups may have anomalous pK_a values in the native state, due to their participation in electrostatic interactions within the domain structure [12]. The considerable fall in ΔG_{unf} values between pH* 4.5 and 1.0 indicates that this type of interaction plays a very important role in the stability of this domain.

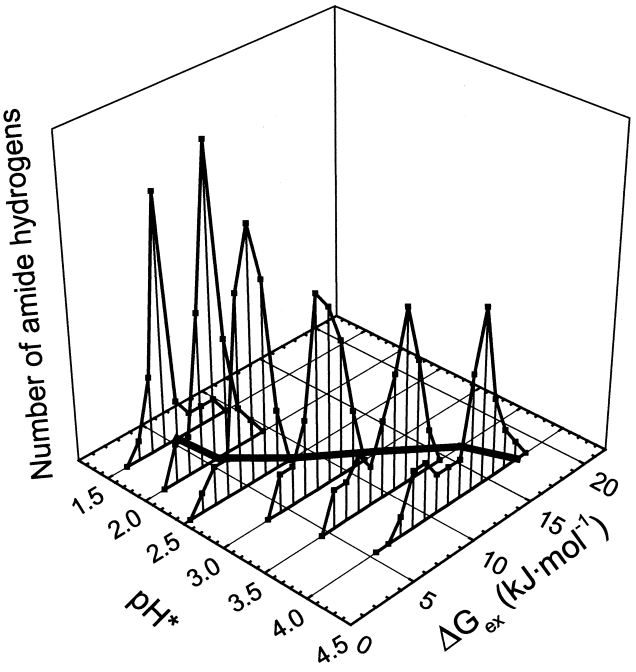

Fig. 2. Distributions of the Gibbs energies of exchange, ΔG_{ex}, at different pH* values. The distributions have been calculated by a frequency count of ΔG_{ex} values using intervals of 1 kJ mol^{-1}. The pH* dependence of the global unfolding Gibbs energy of SH3, corrected for proline isomerization, is shown in continuous line.

For any general equilibrium coupled to protonation the slope of the dependence of ΔG upon pH follows the equation [25]:

$$\left(\frac{\partial \Delta G}{\partial \mathrm{pH}}\right) = 2.303RT\langle \Delta \nu \rangle \tag{2}$$

where $\langle \Delta \nu \rangle$ is the average uptake of protons coupled with the process. For SH3 at pH* 3.0, an average uptake of 1.2 protons occurs upon global unfolding [12]. We shall refer to this value as $\langle \Delta \nu \rangle_{unf}$.

The pH* dependence of ΔG_{ex} for each amide hydrogen can be analyzed by starting from the definition of the opening equilibrium constant, K_{op}, for the amide hydrogen of a particular residue j in terms of probabilities of conformational states, P_i [9–11]:

$$K_{op,j} = \frac{\sum_{i(j-\mathrm{ex})} P_i}{\sum_{i(j-\mathrm{nex})} P_i} = \frac{\sum_{i(j-\mathrm{ex})} \exp\left(-\frac{\Delta G_i}{RT}\right)}{\sum_{i(j-\mathrm{nex})} \exp\left(-\frac{\Delta G_i}{RT}\right)} \tag{3}$$

where ΔG_i represents the Gibbs energy of each state compared to the native state. The sums (j−ex) and (j−nex) are applicable to all the exchange-competent and non-exchange-competent states respectively for the amide group j. Using Eq. 1, the apparent Gibbs energy of exchange for residue j is:

$$\Delta G_{ex,j} = -RT\left[\ln \sum_{i(j-\mathrm{ex})} \exp\left(-\frac{\Delta G_i}{RT}\right) - \ln \sum_{i(j-\mathrm{nex})} \exp\left(-\frac{\Delta G_i}{RT}\right)\right] \tag{4}$$

The derivative of $\Delta G_{ex,j}$ with pH at constant temperature is:

$$\left(\frac{\partial \Delta G_{ex,j}}{\partial \mathrm{pH}}\right)_T = \frac{\sum_{i(j-\mathrm{ex})} \frac{\partial \Delta G_i}{\partial \mathrm{pH}} \cdot \left[\exp\left(-\frac{\Delta G_i}{RT}\right)\right]}{\sum_{i(j-\mathrm{ex})} \exp\left(-\frac{\Delta G_i}{RT}\right)} - \frac{\sum_{i(j-\mathrm{nex})} \frac{\partial \Delta G_i}{\partial \mathrm{pH}} \cdot \left[\exp\left(-\frac{\Delta G_i}{RT}\right)\right]}{\sum_{i(j-\mathrm{nex})} \exp\left(-\frac{\Delta G_i}{RT}\right)} \quad (5)$$

and using Eq. 2 it follows that:

$$\left(\frac{\partial \Delta G_{ex,j}}{\partial \mathrm{pH}}\right)_T = 2.303RT\left[\frac{\sum_{i(j-\mathrm{ex})} \Delta\nu_i \cdot P_i}{\sum_{i(j-\mathrm{ex})} P_i} - \frac{\sum_{i(j-\mathrm{nex})} \Delta\nu_i \cdot P_i}{\sum_{i(j-\mathrm{nex})} P_i}\right]$$

$$= 2.303RT\left[\langle\Delta\nu\rangle_{(j-\mathrm{ex})} - \langle\Delta\nu\rangle_{(j-\mathrm{nex})}\right] = 2.303RT\langle\Delta\nu\rangle_{\mathrm{ex},j} \quad (6)$$

In this equation $\Delta\nu_i$ is the proton uptake coupled with a conformational fluctuation from the native state to state i. Accordingly, $\langle\Delta\nu\rangle_{(j-\mathrm{ex})}$ is the average uptake of protons coupled to the equilibrium between the native state and all the exchange-competent states for residue j. $\langle\Delta\nu\rangle_{(j-\mathrm{nex})}$ is the same average for the non-exchange-competent states. Therefore, the difference between these two quantities, $\langle\Delta\nu\rangle_{\mathrm{ex},j}$, represents the average net uptake of protons coupled with the whole conformational equilibrium exposing the amide hydrogen j to the solvent. According to Eq. 6, the slope of the experimentally determined pH* dependence of the ΔG_{ex} values provides residue-specific information about the influence of the conformational fluctuations of the protein on the apparent pK_a of the carboxylic groups, and therefore on the electrostatic interactions that stabilize the native structure.

Fig. 3A represents the $\langle\Delta\nu\rangle_{ex}$ values calculated at pH* 3.0 in color codes using a cartoon of the SH3 crystal structure [26]. A relatively large set of residues belonging to the domain core, located mainly in the middle of the β-sheets and at the 3_{10} helix, shows $\langle\Delta\nu\rangle_{ex}$ values close to $\langle\Delta\nu\rangle_{unf}$. Other residues near to or within the loops, turns and chain ends have lower $\langle\Delta\nu\rangle_{ex}$ values. When interpreting these results it must be emphasized that at pH* 3.0 all the ΔG_{ex} values for the amide hydrogens of SH3 are appreciably lower than those of ΔG_{unf} (cf. Fig. 1). This is particularly evident for Val9, Leu10, Met25, Leu31, Thr32 and Phe52.

The fact that the ΔG_{ex} values are lower than ΔG_{unf} has been explained in the literature on the basis of the assumption that the exchange of any particular residue would occur through a mixture of two discrete processes, i.e. global unfolding and a transient local fluctuation of the native fold, affecting only a few residues and allowing the exchange catalyzers access to the amide group. This is known as the two-process model for HX [27]. A more general model assumes that under native conditions a protein behaves as an ensemble of conformational states with a broad distribution of Gibbs energies, so that many different states of a protein might lead to the exchange of any particular residue [9–12]. In both models ΔG_{ex} would depend upon the statistic weights of each exchange-competent state according to Eq. 4.

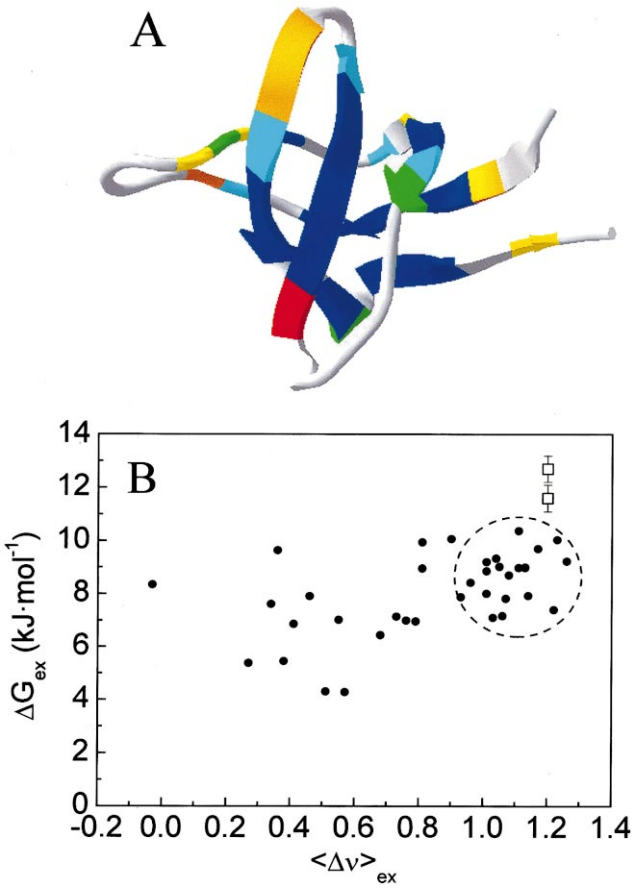

Fig. 3. A: Schematic representation of the SH3 crystal structure showing the average uptake of protons, $\langle\Delta\nu\rangle_{ex}$, coupled with the HX of each residue of SH3. Values are represented in color codes: blue, ≥1.0; cyan, 0.8–1.0; green, 0.6–0.8; yellow, 0.4–0.6; orange, 0.2–0.4: red, <0.2. For residues in white $\langle\Delta\nu\rangle_{ex}$ could not be determined. B: Plot of ΔG_{ex} versus $\langle\Delta\nu\rangle_{ex}$ for the HX of SH3 at pH* 3.0. Filled circles represent the data for each residue. Open squares represent the ΔG_{unf} and $\langle\Delta\nu\rangle_{unf}$ values obtained by DSC, both uncorrected and corrected for proline isomerization effects. The dashed circle encloses the data for a group of residues of the domain core, which exchange by similar unfolding processes (see text).

Taking Met25 as an example, with $\Delta G_{ex} = 8$ kJ mol^{-1} and $\langle\Delta\nu\rangle_{ex} = 1.0$, if this core residue exchanges according to the two-process model, using Eq. 4 one can predict a Gibbs energy of 8.7 kJ mol^{-1} for the local fluctuation. Assuming that the local fluctuation leaves the rest of the protein structure and therefore most of its electrostatic interactions almost unaffected, its $\Delta\nu$ value would be close to zero. The observed $\langle\Delta\nu\rangle_{ex}$ for Met25 would be the weighted average between zero for the local fluctuation and 1.2 for the global unfolding. This gives $\langle\Delta\nu\rangle_{ex} = 0.3$, which is a long way from the observed value of 1.0. This discrepancy also pertains to the rest of the core residues, indicating that the HX of the core residues in the domain does not necessarily involve local fluctuations and global unfolding alone but might also entail partial unfoldings accompanied by protonation changes similar to those accompanying the global unfolding. These unfoldings, therefore, appear to consist of extensive disruptions of the tertiary structure of the domain.

Other residues have lower $\langle\Delta\nu\rangle_{ex}$ values even though their ΔG_{ex} values are not particularly low at pH 3.0. Examples of these are Leu8, Ile30, Arg49 and Gln50. For this type of res-

idue the exchange is dominated by fluctuations of relatively high Gibbs energy although weakly coupled to protonation changes, indicating that they do not affect a substantial part of the electrostatic interactions of the native structure and could be considered to be local unfoldings. One extreme example of this behavior is Trp41 with $\langle \Delta\nu \rangle_{ex} = 0$; its amide hydrogen is buried from the solvent by the side chain of Asn38, with which it forms a hydrogen bond. The ΔG_{ex} value of 8.3 kJ mol^{-1} appears to correspond to a very localized fluctuation, which breaks the hydrogen bond and perhaps affects only the Asn38 side chain.

Looking at Fig. 3A, we can picture the SH3 domain at pH* 3.0 as being composed of a core region, which is exposed to the solvent by large conformational changes involving most of the domain's tertiary structure, surrounded by small subdomains, which can fluctuate independently of the rest.

Fig. 3B depicts ΔG_{ex} versus $\langle \Delta\nu \rangle_{ex}$. The wide scattering of the dots in this plot indicates that many structurally and energetically heterogeneous conformational states are involved in HX in the SH3 domain at pH* 3.0. This is consistent with previous publications showing how native HX patterns can be predicted theoretically for this and other proteins by taking them to be statistical ensembles of conformational states [9–12]. Experimental evidence has also been published to support the opinion that native state HX occurs via a distribution of conformational states rather than discrete unfolding events. Robertson and co-workers [28,29] showed that the processes responsible for the HX of the most protected residues of the ovomucoid third domain occur via a number of unrelated or only partially correlated unfolding motions. Additionally, Parker and Marqusee [30] have proposed for ribonuclease HI and T4 lysozyme that the partially unfolded forms responsible for native state HX are actually a distribution of states rather than discrete phase-separated thermodynamic states. These lines of evidence support the idea of proteins under native conditions as being dynamic systems undergoing a whole variety of conformational changes.

It is interesting, however, that the data in Fig. 3B for the core residues of SH3 are somehow grouped within a relatively small region. This suggests that the unfolding processes which expose these residues to the solvent resemble each other in both their energetics and their influence on the electrostatic interactions of the domain structure. This might be considered as being an indication of partial co-operativity among the exchanges of these residues, although further evidence is needed to check this hypothesis.

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