Native-state HX

Fersht and co-workers[1,2] question the ability of equilibrium hydrogen exchange (HX) methods to provide information about protein-folding intermediates and pathways, citing both theoretical and experimental objections. They stress the principle that an equilibrium measurement 'by itself' cannot prove a kinetic sequence. I agree with the key phrase: 'by itself'. Fersht *et al.* also note that their HX studies of barnase and chymotrypsin inhibitor 2 (CI2) did not yield results comparable to those we obtained for cytochrome *c*.

I view the HX option as follows. The classical view of protein folding visualizes metastable partially unfolded forms as defined intermediates in a defined pathway. By contrast, the so-called 'new view' of protein folding[3,4] visualizes an unlimited number of paths defined by a continuum of partially folded protein forms. Other hypotheses might be formulated. Thermodynamic principle asserts that such intermediates, whatever their character, must exist as high-energy forms of the protein – even under native conditions. The native-state HX method now provides a way of examining the excited-state manifold between the native and unfolded protein, and distinguishing these or other alternatives.

In our initial work on cytochrome *c,* we detected three distinct intermediates[5]. Does this result, by itself, allow us to order these forms in an on-pathway kinetic folding sequence? Certainly not – but other pertinent evidence is accumulating. (1) The highest-energy partially folded form, in which only the N and C helices are structured, has been identified independently as an early intermediate in kinetic folding by HX pulse labeling[6]. (2) This intermediate is blocked and accumulates as a result of the misfolding of a segment that the native-state experiment identifies as the next unit required for folding[7]. (3) Recent work in our lab supports the ordering of the intermediates detected into a sequential unfolding–refolding pathway[8]. (4) The native-state HX method has also identified two high-energy partially unfolded forms in RNase H (Ref. 9), three in apocytochrome b_{562} (using both denaturant and pressure as perturbants)[10,11] and one in a thermophilic rubredoxin[12]. (5) For RNase H, impressive kinetic and molten-globule evidence connects the intermediates with steps in a refolding pathway[13,14].

Fersht and co-workers[1,15] studied barnase and CI2 by straightforward HX methods and found no evidence for folding intermediates, even though a barnase intermediate had been seen by other methods. Under the stable conditions used, local fluctuations dominated exchange and masked the possible role of larger unfoldings, as in most proteins. This is why it was necessary to invent the native-state HX method[5]. The method uses low concentrations of denaturant to stabilize unfolded forms selectively on the basis of the proportion of surface these forms expose. The high-free-energy unfolded forms then descend the energy scale, surpass the local fluctuations, come to dominate the exchange of the many hydrogens that they expose, and can thereby be detected and characterized in favorable cases.

More-recent work with CI2 (Ref. 16) and barnase[2] used the native-state HX method but still failed to find large unfolding intermediates. Partially folded intermediates might not exist in this case, or they might elude HX detection[1,2]. Alternatively, these proteins might not be favorable cases. We have discussed some of the limitations of the native-state HX method; both protein size and protein stability must be large enough to provide the necessary dynamic range[17]. The small size of the CI2 protein (64 residues) might compromise the native-state HX approach, although the work that has been done does not, as yet, provide enough information for the critical reader to judge. The barnase problem revealed another limitation. Itzhaki *et al.*[16] state that 'the existence of partially folded states could not be addressed by the GdmCl-dependence of exchange because of deviation from EX2'. Fortunately, further work connected the non-EX2 HX behavior with the previously enigmatic barnase folding intermediate[18].

Fersht and co-workers also question the 'last out – first in' hypothesis, which suggests that the last hydrogens to exchange might identify the first parts of a protein to fold. This hypothesis has intrigued me since Kallenbach and I initially considered it many years ago[19,20]. We now think that the hypothesis, as stated, cannot be valid in general: the kinds of HX data that we relied on, and those that Woodward and her colleagues[21,22] marshal in defense of the hypothesis, are dominated by local fluctuations that involve the motion of only one or two amino acid residues[23]. These kinds of data can be informative but cannot usefully describe kinetic folding intermediates or pathways. However, the native-state HX method can now characterize the more sizeably unfolded states and therefore makes a real test of the hypothesis possible.

The partially unfolded forms themselves reveal a new dimension of submolecular cooperativity. I suspect that the study of these previously unknown high-energy forms will provide fundamental insights into protein stability, design, cooperativity and even biological evolution, as well as kinetic folding pathways. Accumulating evidence seems to us to be wonderfully encouraging. Others will disagree. Let us go forward and see.

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 0968–0004/98/$19.00 PII: S0968-0004(98)01281-X