LETTERS

Native-state HX

Fersht and co-workers\textsuperscript{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 chromotrypsin inhibitor \(2\) (CI\(2\)) 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\textsuperscript{3} 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 exacted-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\textsuperscript{4}. Does this result, by itself, allow us to order these forms in an on-pathway kinetic folding sequence? Certainly not – but it is the right starting point in 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 cytochrome folding by HX pulse labeling\textsuperscript{4}. (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\textsuperscript{5}. (3) Recent work in our lab supports the ordering of the intermediates detected into a sequential unfolding-refolding pathway\textsuperscript{6}. (4) The native-state HX method has also identified two high-energy partially unfolded forms in \(\text{RNase H}\) (Ref. 1), three in apocytochrome \(c\) \(_{\text{ox}}\) (using both denaturant and pressure as perturbants)\textsuperscript{7,8} and one in a thermophilic rubredoxin\textsuperscript{9}. (5) For \(\text{RNase H}\), impressive kinetic and melo-thion-globose evidence connects the intermediates with steps in a refolding pathway\textsuperscript{10,11}. Fersht and co-workers\textsuperscript{12} studied barnase and CI\(2\) 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\textsuperscript{12}.

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-energy unfolded forms then descend the energy scale, so the free energy of unfolding is a measure of denaturation. As expected, and can thereby be detected and characterized in favorable cases. More recent work with CI\(2\) (Ref. 16) and barnase\textsuperscript{17} 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\textsuperscript{18}. Alternatively, these proteins might not be favorable cases. We have discussed some of the limitations of the native-state HX method, but other pertinent evidence is accumulating. The method uses low concentrations of denaturant and pressure as perturbants\textsuperscript{10,11} and one in a thermophilic rubredoxin\textsuperscript{9}.

Fersht and co-workers\textsuperscript{12} 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\textsuperscript{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\textsuperscript{21,22} have interpreted in defense of the hypothesis, are dominated by local fluctuations that involve the motion of one or two amino acid residues\textsuperscript{21,22}.

We cannot invent the native-state HX method; both protein size and protein stability must be large enough to provide the necessary dynamic range\textsuperscript{7}. The small size of the CI\(2\) protein (64 residues) could be addressed by the Gm\(c\)-dependence of exchange because of deviation from EX\(2\). Fortunately, we further work connected the non-EX\(2\) HX behavior with the previously inorganic chemical fraction intermediate\textsuperscript{23}.

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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 perturbants\textsuperscript{10,11} and one in a thermophilic rubredoxin\textsuperscript{9}.

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