For these reasons, it is unlikely that the unfolding approach will provide new insight into the still enigmatic process of protein folding.

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FERSHT *ET AL.* REPLY—Any novel analysis will, and should, attract intense scrutiny. All of the points that have been raised have precedents, but we welcome the opportunity to discuss them. The analysis of a forward pathway by studying the reverse, for example, is well-established in physical chemistry and enzymology[7] and in protein folding[8].

First, the use of hypothetical thermodynamic cycles is perfectly valid and is frequently employed in thermodynamic analysis; for example, the classic Born–Haber cycle described in most elementary physical chemistry texts. This is allowed by the first law of thermodynamics, which states that energy changes depend only on initial and final states and not on the pathway between them. Cycles equivalent to those we use[1] are also used by the theoreticians for their free-energy calculations (see, for example, ref. 9).

Second, the data analysis is not based on the assumption that barnase exists in only two distinct conformational states. For the equilibrium unfolding cycle, the analysis is based simply on the energy difference between initial (folded) and final (unfolded) states and the first law of thermodynamics. Just as the law allows us to add hypothetical intermediates, it allows real ones to be ignored. For the kinetic cycle, as explained in the article[1], transition-state theory when applied to the unfolding kinetics avoids the problems of intermediates accumulating, because this happens before the rate-determining step only on the refolding pathway. Incidentally, microcalorimetry is not the only way of detecting folding intermediates. Kinetics can detect intermediates that are not observed by equilibrium microcalorimetry and, from measurements of both folding and unfolding rates, either show that a two-state transition is obeyed or that an intermediate accumulates (ref. 10, and our unpublished work). We did indeed, detect an intermediate on the refolding pathway of barnase by kinetic methods and H–D exchange (see refs 2, 3), but its presence does not affect our analysis of unfolding as it occurs after the rate-determining step in the unfolding direction and accumulates only slightly in the transition region (unpublished data).

Third, we stated clearly that microscopic reversibility applies under identical reaction conditions and for a reversible process (ref. 1; see also ref. 7, p. 89–90). It holds not just for "strongly denaturing conditions" but for all concentrations of urea, because folded and unfolded protein always exist in equilibrium even though the equilibrium constant may be far from unity. At any one of these concentrations, the transition states for folding and unfolding are the same. The question is whether or not the transition state changes with change of denaturant. Creighton[11] has summarized the evidence that the nature of the transition state for folding–unfolding does not change with denaturant concentration — the rate of unfolding changes uniformly with changing conditions but refolding changes non-uniformly because the nature of the unfolded state changes[8]. (This is a principal reason why unfolding kinetics is easier to analyse.)

This laboratory has espoused the need for determining the full free-energy pathways of reactions by measuring rate constants in both the forward and reverse directions (see studies on the tyrosyl-tRNA synthetase). We have measured the rate constants for refolding of barnase and many of its mutants and combined these with the unfolding data to characterize the structure of the folding intermediate and the transition states by extension of our protein engineering analysis (manuscript in preparation). The combined data give new information on the folding intermediate but no more information on the transition state than did the unfolding data alone.

Creighton has stated: "Instead of searching for nucleation sites in unfolded proteins, it might be more relevant to search for unfolding nucleation events in the native conformation" (ref. 11). For this reason, and for the necessity of constructing free-energy profiles, it is an essential element of folding studies to measure unfolding rate data. Furthermore, because refolding kinetics is fraught with complications arising from the presence of folding intermediates, *cis–trans* isomerization of prolines, and chemical processes that occur when the denatured protein is incubated for many minutes, unfolding kinetics is simpler to analyse and far less prone to artefact. Unfolding studies by themselves can be used to define the last transition state on the refolding pathway. Unfolding studies might also be more relevant than refolding for relating to phenomena *in vivo,* because the pathway of folding *in vivo* depends on the sequential formation of proteins during translation and may be aided by chaperones, whereas unfolding does not depend on these factors. Far from the unfolding approach being unlikely to provide new insight into protein folding, it should be clear from the above that unfolding studies are an essential element in the understanding of folding, and are just as important as refolding studies.

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Who's who?

SIR—The Haldane beetle story alluded to by Reg Passmore[1] and previous writers belongs to J. B. S., who made the remark in 1951 in a lecture on the biological problems of space flight to the British Interplanetary Society, whose journal then reported it[2] (as discussed in my book *Comparative Social Recognition*[3]).

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SIR—The Haldane beetle story[4] was an invention by Hutchinson to tease Haldane; J. B. S. Haldane was the Haldane referred to and the story has no connection whatever with either Jowett or Oxford. The apocrophyl conversation would have taken place, if it had happened, in a common room in University College, London.

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