is absorbed in the atmosphere, the NIR water vapor bands.

Thus, we see no way of defending Stephen's statement that conclusions based on broad-band measurements (*1*) are inconsistent with other existing data sets where discrepancies were found to occur between measurements in spectral bands and theory. Instead, the broad-band solar absorption findings presented in our report (*1*) are entirely consistent with existing spectral data sets, as both show cloud absorption to be greater than the magnitude predicted by theory.

Stephens makes another critical error when determining cloud absorption with the use of results shown in figure 6 of our report (*1*). Stephens determined cloud absorption, A, from the relationship $A + R + T = 1$, where R is reflectance and T is transmission, and he states that figure 6 in our report (*1*) indicates that a cloud with reflectance (albedo) 0.45 would absorb 0.40. His error is in assuming A occurs entirely in cloud. He does not account for the absorbing surface or for the lower 10 km of the atmosphere as well. An appropriate relationship between R and A is $R + A = 1$, where A now is the fractional absorption by the combined atmosphere-surface system (*7*). The analysis shown in figure 6 in our report is not by itself sufficient to determine cloud absorption; again we would refer the reader to figure 2 in our report (or Fig. 1 here). Consequently, contrary to Stephens erroneous conclusion, in our report (*1*) figure 1 is entirely consistent with figure 6 in our report, as those figures represent alternative ways of viewing the same data. The underlying theme is that observations cannot be reconciled with theory, regardless of the units or method of formulating cloud absorption.

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REFERENCES AND NOTES

1. P. Pilewskie and F. P. J. Valero, *Science* **267**, 1626 (1995).
2. S. Twomey, *J. Atmos. Sci.* **33**, 1087 (1976); G. L. Stephens, *ibid.* **36**, 2111 (1978).
3. The new approach to measure atmospheric absorption during TOGA-COARE and CEPEX evolved from a decade long airborne radiation measurement program; see F. P. J. Valero *et al.*, *Appl. Opt.* **21**, 831 (1982); *Geophys. Res. Lett.* **10**, 1184 (1983); *ibid.* **11**, 465 (1984).
4. T. Hayasaka *et al.*, *J. Appl. Meteorol.* **34**, 1047 (1995).
5. S. A. Ackerman and S. K. Cox, *ibid.* **20**, 1510 (1981).
6. G. L. Stephens and S. Tsay, *Q. J. R. Meteorol. Soc.* **116**, 671 (1990).
7. The effects of our observations beneath cloud being at 10 km instead of at the surface were discussed in our report (*1*) and shown to be consistent with the results of Cess *et al.* (*8*).
8. R. D. Cess *et al.*, *Science* **267**, 496 (1995).

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Interhelical Salt Bridges, Coiled-Coil Stability, and Specificity of Dimerization

In their report (*1*), Kevin J. Lumb and Peter S. Kim address the contribution to the stability of the GCN4 leucine zipper of interhelical salt bridges between ionizable side chains at positions **e** and **g** in the heptad repeat denoted *gabcdef* (residue i in chain 1 with residue i′ + 5 in chain 2, **g** to **e**′). With the use of ^{13}C–nuclear magnetic resonance spectroscopy (NMR), they measured the pK_a (where K_a is the acidity constant) values of two pairs of Glu side chains potentially involved in interhelical salt bridges with Lys side chains in a synthetic model of the homodimeric leucine zipper of GCN4 (GCN4-p1).

Lumb and Kim (*1*) state that potential salt bridges between $Glu^{22,22'}$ and $Lys^{27',27}$ do not contribute to the stability of GCN4-p1 and that potential salt bridges between $Glu^{20,20'}$ and $Lys^{15',15}$ are destabilizing relative to alternative neutral-charge interactions. They conclude that i to i′ + 5 interhelical salt bridges will not necessarily contribute favorably (and in some cases will be unfavorable) to coiled-coil stability and dimerization specificity. They suggest that if there was a favorable electrostatic interaction in the folded GCN4-p1, the pK_a of Glu side chains should have been lower than in the unfolded form.

Lumb and Kim's interpretation of their results is not consistent with the experimental findings that charged side chains at these positions have been shown to play a key role in dimerization specificity (heterodimer formation) of Fos-Jun leucine zippers and de novo designed coiled coils (*2*). Electrostatics have also been shown to control chain orientation (parallel versus antiparallel) in model coiled coils (*3*). Destabilization of homodimers by side chains bearing like charges at these positions is the mechanism proposed to favor heterodimer formation where potential interhelical i to i′ + 5 salt bridges can form (*2*, *3*).

Double-mutant cycle analyses carried out in our laboratory on designed coiled coils have shown that the net electrostatic contribution per interhelical Glu-Lys salt bridge is −0.4 kcal/mol (*4*) and +0.5 kcal/mol per Glu-Glu repulsion (*5*).

Because $Glu^{22,22'}$ are involved in hydrophobic interactions with $Val^{23',23}$ through their methylenes [see figure 2C of (*1*)], the solvent accessibly of the charged carboxylate should accordingly be, on a time average, lower in the folded than in the unfolded form. Therefore, in the folded form of GCN4-p1, $Glu^{22,22'}$ will likely suffer a decrease in solvation free energy (positive $\Delta\Delta G$ solvation). Unless there is strong evidence that this likely loss of solvation free energy is exactly compensated by a negative $\Delta\Delta G_{dipole}$ term or that both of these terms are equal to zero, one cannot conclude that there is no favorable contribution to the stability of GNC4-p1 from electrostatic interactions (negative $\Delta\Delta G$ int) arising from the putative salt bridges between $Glu^{22,22'}$ and $Lys^{27',27}$ on the basis of no change in the pK_a of $Glu^{22,22'}$. On the other hand, hydrophobic interactions involving $Glu^{22,22'}$ in the folded form could play a crucial role in the net electrostatic contribution of these salt bridges to coiled-coil stability. A decrease in solvation free energy of the charged carboxylates of $Glu^{22,22'}$ in the folded form could be compensated for by the formation of hydrophobic interactions (involving the methylenes of $Glu^{22,22'}$ and $Val^{23',23}$ side chains at positions **a**), leaving a net stabilization provided by purely electrostatic interactions between Glu and Lys side chains at neutral pH, as determined by double-mutant cycle analysis on de novo designed coiled coils in our laboratory (*4*).

Lumb and Kim (*1*) state that when $Glu^{20,20'}$ are replaced by Gln residues, the stability of GCN4-p1 is increased. To explain this result, they propose that Gln side chains pack more efficiently than Glu side chains at the dimer interface. This is more a consequence rather than an explanation. The solvation free energy of a neutral polar group is about 60 kcal/mol less than that of a charged group (*6*). Therefore the fact that the $Gln^{20,20'}$ analog of GCN4-p1 is more stable likely results from a significantly lower cost of solvation free energy upon packing at the dimer interface. Moreover, replacing the charged carboxylate by a neutral carboxamide group could alleviate any destabilizing interaction of the charged carboxylate with polar groups in its surrounding at the dimer interface. Both effects should allow a stronger net contribution of hydrophobic interactions at the dimer interface from hydrophobic moieties of

$Gln^{20,20'}$ as compared to $Glu^{20,20'}$.

Also based on the increased stability of the $Gln^{20,20'}$ GCN4-p1 analog, Lumb and Kim (*1*) conclude that interhelical charge-charge interactions should not be weighted more heavily than potential neutral-charge interactions at i and i′ + 5 positions in the prediction of coiled-coil dimerization specificity. Strictly speaking, this conclusion is only relevant to the stability of the particular case of GCN4-p1. To make it a general statement on coiled-coil stability, a similar trend should be observed for a $Gln^{22,22'}$ GCN4-p1 analog. No such result is presented by Lumb and Kim (*1*). On the other hand, work carried out in our laboratory on designed coiled coils with an entirely hydrophobic dimerization interface (positions **a** and **d**) has shown that potential interhelical *i* and *i′* + 5 Gln-Lys interactions provide 0.15 kcal/mol (per pair) less stabilization free energy than Glu-Lys interactions (*4*).

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REFERENCES AND NOTES

1. K. J. Lumb and P. S. Kim, *Science* **268**, 436 (1995).
2. E. K. O'Shea *et al.*, *ibid.* **245**, 646 (1989); E. K. O'Shea *et al.*, *Cell* **66**, 699 (1992); N. E. Zhou, C. M. Kay, R. S. Hodges, *J. Mol. Biol.* **237**, 500 (1994).
3. O. D. Monera *et al.*, *J. Biol. Chem.* **268**, 19218 (1993); O. D. Monera, C. M. Kay, R. S. Hodges, *Biochemistry* **33**, 3862 (1994).
4. N. E. Zhou, C. M. Kay, R. S. Hodges, *Protein Eng.* **7**, 1365 (1994).
5. W. D. Kohn, C. M. Kay, R. S. Hodges, *Protein Sci.* **4**, 237 (1995).
6. A. Warshel and S. T. Russell, *Q. Rev. Biophys.* **17**, 283 (1984).

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Response: Basic-region leucine zipper (bZIP) transcription factors bind to DNA as homo- or heterodimers, and the specificity of dimerization resides in the leucine-zipper region, a parallel, two-stranded coiled coil (*1*). Since 1972, interhelical salt bridges in coiled coils have been assumed to be important for stability and dimerization specificity [see (*2*)]. Our study of the GCN4 leucine zipper (*2*) challenges this long-held assumption and will naturally attract scrutiny. We welcome the opportunity to discuss the comments raised by Hodges and co-workers. In particular, it is important to distinguish between pK_a measurements and double-mutant cycle analyses.

First, although there are several factors that affect pK_a values in proteins (*3*), the difference in pK_a between the folded and unfolded states provides a *direct* measure of the contribution of an ionizable group to the free energy of folding (ΔG_f). This statement is a consequence of thermodynamics (*3, 4*). If a negatively charged residue contributes favorably to ΔG_f, the pK_a will be lower in the folded than in the unfolded state (Fig. 1). A classic example of a salt bridge stabilizing a protein and thereby reducing a pK_a is the activating salt bridge of chymotrypsin; a dramatic example is the buried salt bridge in T4 lysozyme (*5*).

Values of pK_a can be measured accurately using the ^{13}C-NMR method described in our report (*2*). In particular, pK_a value for the unfolded-state was measured rather than estimated. These pK_a measurements indicate unambiguously (*2*) that the negatively charged Glu residues that form salt bridges in the crystal structures of the GCN4 leucine zipper [figure 2A in (*2*); see also (*6, 7*)] do not make favorable contributions to ΔG_f, relative to the corresponding situation in which the Glu residues are neutral (protonated).

Second, although it is possible that the situation differs in solution, our study focused on residues that actually form salt bridges in high-resolution x-ray crystal structures (*6, 7*). In contrast, salt bridge formation has been *assumed* in the double-mutant cycle studies referred to by Hodges *et al.* (*8*). There are numerous examples of potential salt bridges that do not actually form in crystal structures of various leucine zippers (*6, 7, 9*).

Third, contrary to the statements of Hodges *et al.*, double-mutant cycle studies do not necessarily measure electrostatic interactions (*10*). In a double-mutant cycle, the residues of interest are mutated separately and together to measure the free energy of interaction between the two residues of interest, called the coupling energy (*10, 11*). The essential point is that the coupling energy will contain contributions from *all* interactions between the residues of interest, not just electrostatic. The coupling energy may reflect, for example, contributions from solvation, van der Waals contacts and indirect effects propagated through the structure as a result of the mutations (*10*).

Fersht *et al.* (*10*) have explained succinctly that in order for a coupling energy to constitute a measure of an electrostatic interaction, it must be shown that (i) the nonelectrostatic contributions to the coupling energy in the single mutants are additive in the double mutant and, (ii) there are no conformational changes in the mutant proteins that alter the electrostatic interactions of the unmutated charged residue with the rest of the protein.

To demonstrate that the nonelectrostatic terms are additive, the coupling energy must be shown to be negligible at high ionic strength, where coulombic interactions are screened (*10*). To rule out conformational changes requires high-resolution structural data. Neither of these requirements have been established in the double-mutant cycle studies of putative interhelical salt bridges in coiled coils performed to date (*8, 12*). Thus, although it is possible that the coupling energies obtained in these studies correspond to electrostatic effects, it has not been determined if these studies are measuring only (or even predominantly) the electrostatic interaction between the residues of interest.

Indeed, it seems likely that large, nonadditive free energy terms will result from structural reorganization at the hydrophobic helical interfaces of the mutant coiled coils. In the double-mutant cycle studies of salt bridges in coiled coils reported to date, residues at positions **g′** and **e** are changed to Ala or Gln (*8, 12*). This effective removal of the side chain, especially for the long side chains of Lys and Arg, will almost certainly change the hydrophobic contacts in the interhelical core, which consists of residues at positions **a**, **d′**, **e**, and **g′**, where prime (′) denotes residues of the opposing helix (*6, 7, 9*). Hydrophobic interactions are substantial (*13*) and could easily contribute 1 kcal/mol to the coupling energy. In the absence of high-resolution structural data, one cannot assume that the structural consequences of such indelicate mutations are additive.

The largest favorable coupling energies that have been observed in coiled coils are between Glu and Arg residues in studies of the VBP leucine zipper by Vinson and colleagues [about −1 kcal/mol, relative to Ala

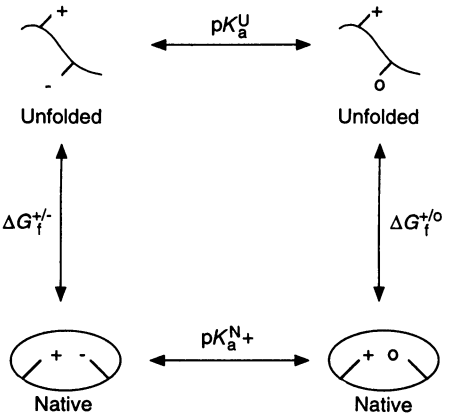

Fig. 1. Thermodynamic cycle showing the relationship (*3, 4*) between pK_a values and the free energy of folding (ΔG_f).

$$\Delta G_f^{+/-} - \Delta G_f^{+/o} = -2.303RT(pK_a^U - pK_a^N)$$

A difference in the free energy of protonation in the native (N) and unfolded (U) states requires a difference in ΔG_f when the ionizable group is charged or neutral (protonated).