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Long-Range, Small Magnitude Nonadditivity of Mutational Effects in Proteins†

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The question of whether multiple mutations within a protein sum additively or nonadditively has fascinated investigators for more than a decade, both because of the potential implications for understanding how proteins function and because of the practical implications for protein engineering. The energetics of interaction between two mutation sites within a protein can be assayed by summing the energetic perturbation caused by each mutation separately and comparing this sum to the energetic perturbation measured in the double mutant. The finding of nonadditivity or an "interaction free energy" between sites (Ackers & Smith, 1985) is most frequently interpreted to indicate either a direct pairwise or a simple triangulated steric contact between the two residues. However, examination of double and multiple mutants in several protein systems (including human hemoglobin, staphylococcal nuclease, tyrosyl-tRNA synthetase, glutathione reductase, subtilisin BPN′, and T4 lysozyme) reveals that small magnitude energetic coupling (i.e., nonadditivity) between sites *that cannot possibly be in direct contact* is a general characteristic of protein systems.

The prevailing interpretive paradigm in double mutant studies holds that residues which are distantly separated within a protein generally exhibit additive energetics. This correlation is empirically based. Interaction free energies of several kilocalories per mole (5−10) constitute "nonadditivity" and are strongly correlated with either a direct steric contact between two residues or a short-range steric interaction involving triangulation through a mediating residue or a substrate molecule [Carter et al., 1984; Wells, 1990; also see Mildvan et al. (1992) for a review of short-range nonadditivities]. Interaction free energies below about 1.0−1.5 kcal/mol are usually considered additive and are correlated with mutations that are separated by larger distances. These correlations have been successfully utilized as an interpretive strategy to probe the active sites of enzymes and to identify residues within a protein which interact during folding [see Wells (1990) and Shortle (1992) for recent reviews]. The interaction free energy between two mutation sites is defined thermodynamically, however, and neither makes any assumptions about the underlying structural mechanism nor *a priori* implies a proximity correlation (Ackers & Smith, 1985).

A number of "enigmatic" cases of nonadditivity have surfaced involving residue pairs separated by large distances (Shortle & Meeker, 1986; Perry et al., 1989; Howell et al., 1990; Robinson & Sligar, 1993). Case-specific mechanisms have been invoked to explain these long-distance nonadditivities. Examination of the patterns of effects of large numbers of mutations throughout a protein, however, often yields information of a different quality than examination of one or even several mutations within a protein [e.g., see Pettigrew et al. (1982), LiCata et al. (1990), Turner et al. (1992), and LiCata et al. (1993)]. In 1990, LiCata et al. presented measurements of interaction free energies in a set of 40 mutant hybrid hemoglobin tetramers. While all of the double mutants examined were separated by distances of 15−30 Å, 55% of the pairs exhibited statistically significant nonadditivity (LiCata et al., 1990). A recent study of 71

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double mutants in the staphylococcal nuclease system (Green & Shortle, 1993) found that 59% of the mutations examined were nonadditive outside error and noncontact. Expansion of our 1990 data set to 67 double mutants in the human hemoglobin system and comparison of these data with the staphylococcal nuclease data, and data on tyrosyl-tRNA synthetase, glutathione reductase, subtilisin BPN′, and T4 lysozyme, reveal common patterns of unidirectional small magnitude nonadditivities between sites in proteins that are distantly separated. Due to the small magnitude of this long-range nonadditivity (generally ≤ 1 kcal/mol), examination of these couplings on an individual basis has generally resulted in their being classified as additive rather than nonadditive.

Quantitating Nonadditivity. Consider two single-site mutations, A and B, within a protein. To assess whether sites A and B energetically interact, one must measure four separate free energies associated with some function of the protein: ΔG_{wt}, the functional free energy in the wild-type molecule; ΔG_A, the functional free energy in the molecule with a mutation at site A; ΔG_B, the functional free energy in the molecule with a mutation at site B; and ΔG_{AB}, the functional free energy in the molecule with simultaneous mutations at sites A and B.

We can then define the perturbation caused by mutation to the function of interest:

$$\Delta\Delta G_A = \Delta G_A - \Delta G_{wt}$$

$$\Delta\Delta G_B = \Delta G_B - \Delta G_{wt}$$

$$\Delta\Delta G_{AB} = \Delta G_{AB} - \Delta G_{wt}$$

The central question of whether mutation sites are additive or nonadditive is

$$\text{Does } \Delta\Delta G_{AB} = \Delta\Delta G_A + \Delta\Delta G_B?$$

By rearranging this question, we can define a measure of the amount of nonadditivity displayed in the system:

$$\Delta\Delta G_{AB} - (\Delta\Delta G_A + \Delta\Delta G_B) = \delta \qquad (1)$$

δ (delta) is the interaction free energy between sites A and B relative to the function of interest. When $\delta = 0$, the sites are additive, and when δ = any quantity, the sites are nonadditive by δ kcal/mol. When more than two mutations are combined within a protein, eq 1 is expanded accordingly.

For in-depth discussions of the theory, assumptions, and experimental considerations that enter into the determination of nonadditivity of mutational effects, the reader is referred to earlier publications [e.g., Ackers and Smith (1985), LiCata et al. (1990), and Mildvan et al. (1992)]. There are, however, two specific issues germane to the present analysis that warrant mention. First, multiple-site mutation interactions are complexed by an issue we have called directionality (LiCata et al., 1990). An interaction free energy (δ) between two or more sites is mutual and provides no information about directionality (i.e., there is no "first" or "second" mutation, etc.). The additivity of mutations A, B, and C, however, may be calculated in several ways: mutants A, B, and C may be measured separately and compared to mutant ABC; the two mutants AB and C may be summed and compared to mutant ABC, etc. Summing a triple mutant in this second manner can impose a directionality on the results

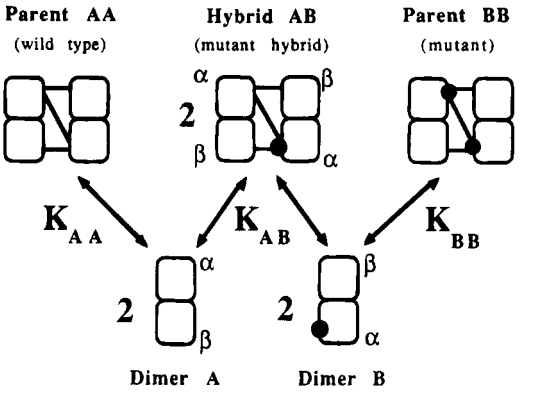

FIGURE 1: Schematic showing the disproportionation reaction used to measure the interaction free energy (δ) in hemoglobin tetramers. The black dots represent mutation sites. Quantitation of the hybrid equilibrium, which simultaneously contains the reference molecule and both the single and double mutant species, allows direct measurement of the nonadditivity [see LiCata et al. (1990) for details].

when it assumes that the interaction free energy between sites A and B does not change in mutant AB versus mutant ABC. Such analyses may also result in some redundancy of information when the same interactions are summed in more than one way. Summation order and directionality considerations can make analysis of multiple mutant data quite complex. For the purposes of the present report, we have used the interaction free energies of multiple mutants exactly as they have been reported in the literature, even though, in a few cases, we would sum the free energies differently. This difference in analytical approach does not alter the conclusions of this study. Second, because the additivity or nonadditivity of a set of mutants is always relative to a particular function of the protein, if one assays a different function, one is essentially asking a new additivity question. Two sites may be additive with respect to some functions and nonadditive relative to others. In several of the protein systems reviewed herein, the same set of double mutants has been functionally assayed in several different ways. An interaction free energy (δ) is therefore separately calculated for each different way of assaying these mutations. This practice is sound (Ackers & Smith, 1985), was used in the original publications of the data reviewed, and is continued herein.

Nonadditivity in Human Hemoglobin. Nonadditivity in human hemoglobin is measured relative to the *function* of dimer to tetramer assembly. Because the hemoglobin tetramer consists of two α and two β subunits, a mutant hemoglobin tetramer will naturally contain two mutation sites (e.g., if the mutation is in the α subunit, both α subunits in the tetramer will contain a mutation site). Dissociation of the tetramers into $\alpha\beta$ dimers and formation of hybrid tetramers allow one to examine the nonadditivity between pairs of mutation sites in the molecule where the mutations themselves are identical (see Figure 1). Therefore, the nonadditivity between sites is the difference between the functional perturbation when both sites are mutated versus twice the single-site perturbation found in the hybrid tetramer:

$$\Delta\Delta G_{AA} - 2(\Delta\Delta G_A) = \delta \qquad (2)$$

Here the perturbation to function caused by mutation is

Table 1: Interaction Free Energies (δ) for Human Hemoglobins in the Unligated, Oxygenated, and CNmet Ligated States[a]

mutation	δ deoxy	δ oxygenated	δ CNmet
Ch (Chesapeake) αN92L	0.06 ± 0.35	0.12 ± 0.1	0.06 ± 0.02
Au (Austin) βR40S	0.43 ± 0.45	0.43 ± 0.46	nd
Ag (Athens, GA) βR40K	0.18 ± 0.1	0.09 ± 0.21	0.27 ± 0.05
Wo (Wood) βH97L	0.06 ± 0.06	0.01 ± 0.05	0.08 ± 0.03
Ya (Yakima) βD99H	0.31 ± 0.1	−0.01 ± 0.1	−0.06 ± 0.03
Ra (Radcliffe) βD99A	0.21 ± 0.1	0.0 ± 0.1	−0.03 ± 0.02
Hd (Hotel Dieu) βD99G	0.26 ± 0.1	0.01 ± 0.1	nd
Ke (Kempsey) βD99N	0.52 ± 0.1	−0.01 ± 0.1	nd
Sm (St. Mande) βN102Y	0.39 ± 0.19	0.9 ± 0.23	0.81 ± 0.1
Dl (Dallas) αN97K	0.08 ± 0.1	0.06 ± 0.1	0.04 ± 0.02
Ta (Tarrant) αD126N	0.12 ± 0.1	−0.08 ± 0.1	−0.05 ± 0.06
Da (Des Arg) αR141 deleted	0.30 ± 0.16	0.25 ± 0.12	0.20 ± 0.08
Le (Legnano) αR141L	0.49 ± 0.05	nd	0.61 ± 0.06
Sc (St. Claude) αK127T	1.35 ± 0.55	0.91 ± 0.43	0.99 ± 0.14
Bu (Bunbury) βD94N	0.08 ± 0.02	0.14 ± 0.03	0.09 ± 0.03
Th (thiomethyl) βC93-tm	0.17 ± 0.11	0.0 ± 0.19	0.1 ± 0.04
Ne (NES) βC93-NES	0.20 ± 0.17	0.19 ± 0.1	0.21 ± 0.05
Dh (Des His) βH146 deleted	0.32 ± 0.08	0.43 ± 0.11	0.23 ± 0.08
Ka (Kariya) αK40E	0.17 ± 0.12	0.08 ± 0.1	−0.01 ± 0.1
Ao ("wild type")	0.14 ± 0.11	0.09 ± 0.1	0.0 ± 0.1
Gn (G-Norfolk) αD85N	0.10 ± 0.04	0.05 ± 0.04	0.04 ± 0.04
Ab (Abruzzo) βH143R	0.21 ± 0.1	0.20 ± 0.1	nd
Tg (TyGard) βP124Q	0.09 ± 0.20	0.05 ± 0.11	0.06 ± 0.03
Sd (San Diego) βV109M	0.07 ± 0.05	0.08 ± 0.04	0.14 ± 0.04

[a] There are 24 unligated mutant pairs, 23 oxygenated mutant pairs, and 20 CNmet ligated mutant pairs. All experiments were performed in 0.1 M Tris, 0.1 M NaCl, and 1 mM EDTA at pH 7.40 and 21.5 °C.

defined as in the general case (e.g., $\Delta\Delta G_A = \Delta G_A - \Delta G_{wt}$); $\Delta\Delta G_A$ is the perturbation free energy in a molecule with a mutation in one α chain (single site), and $\Delta\Delta G_{AA}$ is the perturbation free energy in a molecule with mutations in both α chains (double site).

The technique of quantitative low-temperature isoelectric focusing allows one to directly measure the interaction free energy (δ) between these pairs of mutation sites in hemoglobin (LiCata et al., 1990), thereby actually avoiding much of the error propagation normally involved in such calculations. The interaction free energies for 67 mutation site pairs, along with their experimental errors, are listed in Table 1. An extensive series of controls have been used to establish that the nonadditivity displayed between noncontact sites in hemoglobin is statistically significant (LiCata et al., 1990; LiCata, 1990; Speros et al., 1991). Although more than 100 mutant hybrid tetramers have been examined in the Ackers laboratory, only those where both the singly and the doubly mutated species are in the same state of ligation are included here. This eliminates differential ligation as a possible contributor to the observed nonadditivities.

When the data for human hemoglobin are plotted in a "conventional" manner used to look for nonadditivity (plotting the sum of single-site mutation $\Delta\Delta G$'s versus the double mutant $\Delta\Delta G$'s) [e.g., see Wells (1990)], the data fit a line with a slope of 0.9 and $r^2 = 0.96$. By the criteria of Wells (1990), these mutant pairs would all be classified as additive. While such correlation plots demonstrate that there are no large nonadditivities in these data sets, such as might be expected if two sites are in direct steric or noncovalent contact, they overlook the existence of small magnitude nonadditivities. Figure 2 shows the hemoglobin interaction free energies (δ) plotted with the individual mutations listed on the abscissa. The interactions are all very small in magnitude: only one pair exceeds 1 kcal/mol of nonadditivity, but 68% of the δ's are statistically non-zero. Fifty-two percent of the interactions are nonadditive by more than 0.1 kcal/mol. It is immediately apparent that nearly all the interaction free energies in the hemoglobin system are positive. If these effects were due to random scatter, one would expect approximately equal numbers of positive and negative values.

Nonadditivity in Protein Folding. Interaction free energies between sites during the unfolding of a protein are also calculated using eq 1, where the $\Delta\Delta G$'s of unfolding of the double and single mutants are calculated relative to the unfolding free energy of the wild-type protein, e.g.

$$\Delta\Delta G_A(\text{unfolding}) = \Delta G_A(\text{unfolding}) - \Delta G_{wt}(\text{unfolding}) \quad (3)$$

The interaction free energies for 71 double mutant pairs in the staphylococcal nuclease system relative to denaturation by GuHCl (Green & Shortle, 1993) are plotted in Figure 3A. The pattern of nonadditivities in the staphylococcal nuclease system is quite similar to that seen for hemoglobin. Like the hemoglobin δ's almost all nonadditivities are positive, and here all but five mutant pairs are nonadditive by ≤1.5 kcal/mol. The experimental errors on these data are all ±0.3 kcal/mol, which makes 59% of the δ's nonadditive outside error. Figure 3b shows 20 δ's for the heat denaturation of double, triple, and quadruple mutants in the T4 lysozyme system. Here the nonadditivities are generally quite small, and although the unidirectionality is not as strong, the δ's are mostly negative.

Nonadditivity in Enzyme Catalysis. Nonadditivity in enzymatic systems is commonly measured relative to the change in transition-state stabilization free energy, $\Delta\Delta G_T^{\ddagger}$, which is determined for enzymes containing mutations A, B, and the double mutant AB using the relationship:

$$\Delta\Delta G_{T\ \text{mutant}}^{\ddagger} = -RT \ln [(k_{cat}/K_M)_{mutant}/(k_{cat}/K_M)_{wt}] \quad (4)$$

The interaction free energy (δ) is then calculated using eq 1. Interaction free energies for mutation sites in subtilisin BPN′, tyrosyl-tRNA synthetase, and glutathione reductase are shown in Figure 4. Again, all δ's shown are for sites that do not contact one another. Due to their small magnitude, all of these interactions have previously been classified as additive (Wells, 1990). There is a definite unidirectionality to the observed nonadditivities in all cases. As with the previous cases, this long-range nonadditivity is of small magnitude. In the case of subtilisin BPN′ the nonadditivity appears to accumulate as one increases the number of mutation sites.

DISCUSSION

We have examined published data on *noncontact* mutations in six different protein systems, each with between 14 and 71 double or multiple mutation sets, and have found similar patterns of nonrandom site−site interaction free energies. We have only used data from extensively studied protein systems where a relatively large number of noncontact mutant combinations have been examined. Smaller noncontact multiple mutation data sets which exist for other protein systems [e.g., triose-phosphate isomerase kinetics (Blacklow et al., 1991) and human growth hormone binding

to receptors (Cunningham & Wells, 1991)] also appear to exhibit these nonrandom patterns when viewed in light of the evidence from the large data sets. Very large multiple mutant data sets of complementarity-type assays exist for some protein systems; however, we have only used multiple mutant data where quantitation of the *free energy* of site–site interaction is available.

Although errors have only been published for the hemoglobin and staphylococcal nuclease systems, the finding of similar patterns of small magnitude long-range nonadditivity over such a wide range of systems and functions—including both monomeric and multimeric proteins and relative to the functions of enzyme catalysis, protein folding, and subunit assembly—suggests that such effects are universal. Our examination essentially constitutes a simplified form of meta analysis [e.g., see Research News (1990)]. It is the consistent observation of nonrandom patterns in these data that point toward the general existence of small magnitude nonadditivity between sites in proteins that can in no way directly pairwise interact via steric contact, salt bridges, H-bonding, hydrophobic bonding, or London dispersion forces—and not interaction between a few, isolated sites but between the majority of noncontact sites assayed.

Because these small magnitude, long-range effects are present relative to a wide range of protein functions, either there must be a family of different molecular mechanisms responsible for these effects (i.e., one mechanism relative to catalysis, one relative to protein folding) or more likely there is one general protein property which is manifested when these different systems are examined in these varied ways. Long-range propagation of electrostatic interactions through the low dielectric of the protein is one of the most examined possible mechanisms for long-range nonadditivity (Russell et al., 1987; Jackson & Fersht, 1993; Lowenthal et al., 1993). The nonadditivities observed herein are found between both charge–change site pairs and noncharge–change site pairs, however (e.g., only one of the mutant pairs in the staphylococcal nuclease system involves creation or deletion of an ion pair). Global and local conformational changes could also explain long-range nonadditivity. Small conformational changes are observed in T4 lysozyme as far as 20 Å away from the site of single mutations by crystallography (Alber et al., 1988) and across distances of 15 Å in staphylococcal nuclease by NMR (Hibler et al., 1987). The possibility of small global or local conformational differences which are below the reliable resolution even of structures solved by molecular replacement cannot be ruled out. In protein folding, it has been proposed that interactions among residues which are not in contact in the native state could occur in the denatured state (Shortle, 1992; Green & Shortle, 1993). These proposed mechanisms are somewhat case specific, and no one of them alone can account for the wide range of nonadditivities observed. Although the data examined here allow for little more than mechanistic speculation, it may be that more general molecular mechanisms could be responsible for some of these observations. For example, perturbations in structure which might seem highly localized by crystallographic examination may perturb more global "solid-state" elements of the protein, such as global vibrational modes, which may be important in function, or disruption of global electrostatic fields within the protein. Another possibility is that seemingly inconsequential structural changes within a protein may dramatically alter the distribution of statistical mechanical substates accessible to the system. Redistributions within the ensemble could then be reflected in the observed functional behavior. Current debate among laboratories examining the theoretical basis of component analyses of free energy simulations is beginning to provide a framework for addressing this possibility (Mark & van Gunsteren, 1994; Boresch et al.,

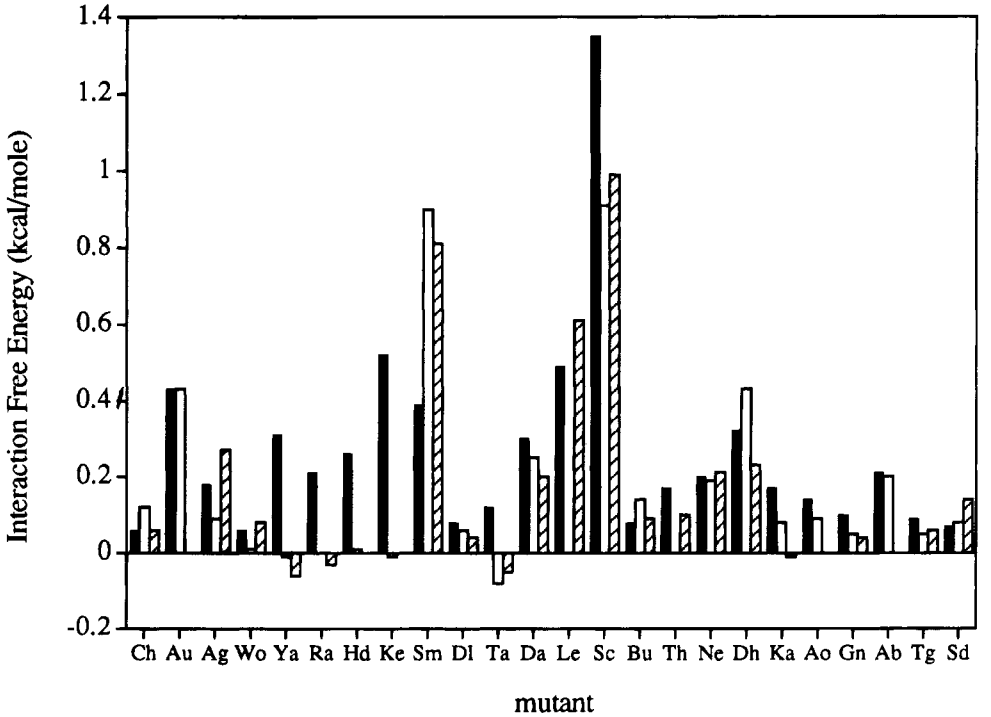

FIGURE 2: Noncontact interaction free energies (δ) between mutant sites in human hemoglobin. The δ for each pair of mutations is shown in three different ligation states: unligated (solid bars), oxygenated (open bars), and cyanomet ligated (striped bars). The name and mutation corresponding to each two-letter code are listed in Table 1. The data are from LiCata et al. (1990, 1993).

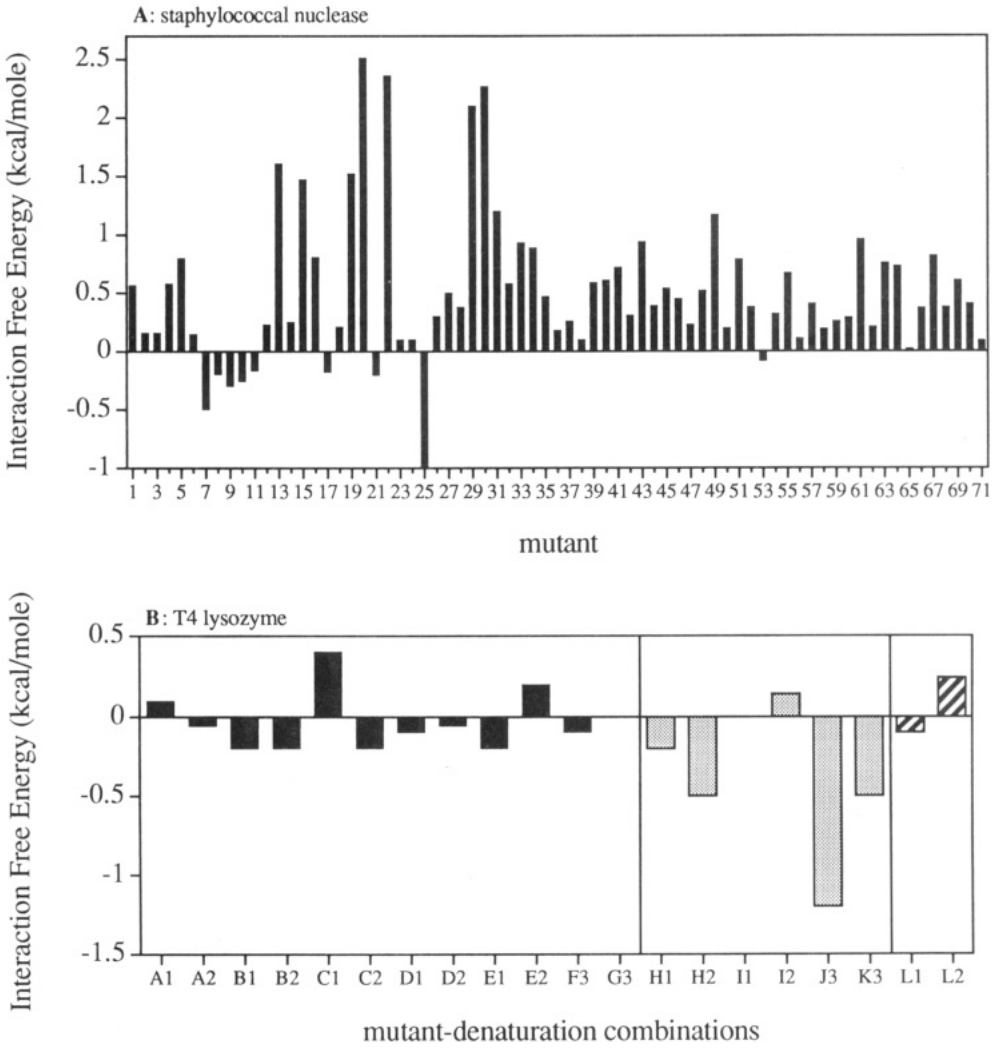

FIGURE 3: Noncontact interaction free energies in two protein folding systems. Staphylococcal nuclease δ's (panel A) are from Green and Shortle (1993). The mutation pairs are (1) 15V+72V, (2) 15V+85V, (3) 15V+113A, (4) 72V+85A, (5) 72V+113A, (6) 85A+113A, (7) 18M+33S, (8) 18M+69T, (9) 18M+90S, (10) 33S+90S, (11) 69T+90S, (12) 7A+23F, (13) 7A+37A, (14) 7A+75V, (15) 7A+79S, (16) 23F+37A, (17) 23F+75V, (18) 23F+79S, (19) 37A+75V, (20) 37A+79S, (21) 37A+117L, (22) 37A+118D, (23) 62A+66L, (24) 62A+88V, (25) 66L+88V, (26) 75V+79S, (27) 75V+117L, (28) 79S+117L, (29) 79D+118D, (30) 79S+118D, (31) 117L+118D, (32) 23F+33S, (33) 23F+69T, (34) 23F+90S, (35) 37A+33S, (36) 37A+69T, (37) 37A+90S, (38) 75V+33S, (39) 75V+69T, (40) 75V+90S, (41) 62G+88V, (42) 7A+15V, (43) 7A+72V, (44) 7A+85A, (45) 7A+92V, (46) 7A+113A, (47) 7A+130G, (48) 23F+15V, (49) 23F+72V, (50) 23F+85A, (51) 23F+92V, (52) 23F+113A, (53) 23F+130G, (54) 37A+15V, (55) 37A+72V, (56) 37A+85A, (57) 37A+92V, (58) 37A+113A, (59) 37A+130G, (60) 75V+15V, (61) 75V+72V, (62) 75V+85A, (63) 75V+92V, (64) 75V+113A, (65) 75V+130G, (66) 79S+15V, (67) 79S+72V, (68) 79S+85A, (69) 79S+92V, (70) 79S+113A, and (71) 79S+130G. T4 lysozyme interaction free energies (panel B) are from the secondary source (Wells, 1990) and from Dao-pin et al. (1991). Black bars are double mutants, shaded bars are triple mutants, and striped bars are quadruple mutants. Code letters correspond to mutants as follows: (A) 16E+119E, (B) 16E+135E, (C) 16E+154E, (D) 119E+135E, (E) 135E+147E, (F) 3C+54V, (G) 3C+54T, (H) 16E+135E+147E, (I) 119E+135E+147E, (J) 3C+54T+96H, (K) 3C+54T+146T, and (L) 16E+119E+135E+147E. Code numbers correspond to the pH of denaturation: (1) pH 2.8, (2) pH 5.3, and (3) pH 6.5.

1994). Further, models for molecular action based on balances among widely delocalized strain forces (Hopfield, 1973) would also tend to predict general nonadditivity of long-range pairwise interactions. Specific experiments will be required that are designed to examine the possible origins of these effects. The recognition of the generality of these effects is only a first step toward understanding what they are telling us about the way proteins work.

The predominance of unidirectional (same sign) interaction free energies within each protein system, while providing the argument that these effects are not simply data scatter, is enigmatic. For hemoglobin and the protein folding systems, positive interaction free energies indicate that the double mutant perturbs the system less than the sum of the two individual mutants (i.e., the mutations are "subadditive"), while negative δ's indicate that the mutations are "superadditive" and perturb the system more than the sum of the two individual mutants. For changes in the transition-state stabilization energy in the enzymatic systems, the opposite relationship holds between the sign of δ and the sub- or superadditivity of mutations. The sign of the long-range nonadditivities observed in these systems is not apparently correlated with the function assayed, with the sign of the original functional free energy, with charge−charge or noncharged interactions, with the creation or deletion of ion pairs, or with the tendency for mutations to be deleterious versus advantageous to function or stability.

As it is presently practiced, protein engineering via multiple-site mutation does not require knowing the exact

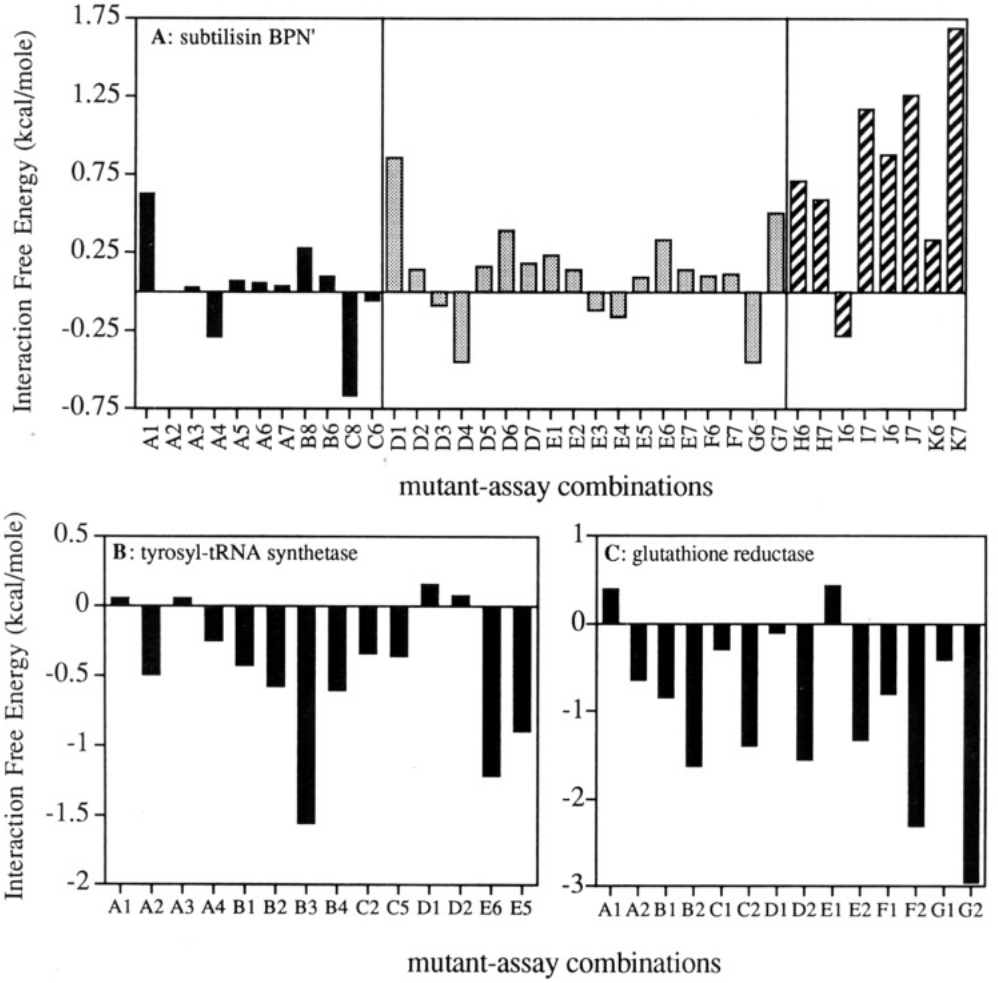

FIGURE 4: Noncontact interaction free energies for three enzymatic systems. Panel A shows δ's for double (black bars), triple and quadruple (shaded bars), and five- and six-site mutants (striped bars) in the subtilisin BPN′ system. Multiple-site mutants in the subtilisin BPN′ system are generally analyzed in binary combinations: e.g., a δ for the multiple-site mutant ABC might be analyzed by examining the combination AB + C; such analyses lead to some redundancy of information (see text). Code letters correspond to mutants as follows: (A) E156S+Y217L, (B) D99S+E156S, (C) D99K+E156K, (D) E156S+Y217L+G169A, (E) (E156S,Y217L)+G169A, (F) G166A+(G169A, E156S,Y217L), (G) G166A+(S24C,H64A), (H) (E156S,G169A,Y217L)+(S24C,H64A), (I) (S24C,H64A,G169A,Y217L)+G166A, (J) (S24C,H64A,G166A)+(E156S,G169A,Y217L), and (K) (S24C,H64A)+(E156S,G166A,G169A,Y217L). Code numbers refer to different substrates assayed: the substrate is succinyl-L-Ala-L-Ala-L-Pro-L-(*x*)-*p*-nitroanilide, where the amino acid *x* is (1) Glu, (2) Gln, (3) Ala, (4) Lys, (5) Met, (6) Phe, or (7) Tyr, or (8) benzoyl-L-Val-Gly-L-Arg-*p*-nitroanilide. Panel B shows δ's for five different double mutants examined for six different activities in the tyrosyl-tRNA synthetase system. Code letters correspond to the mutant combinations: (A) C35G+H48G, (B) C35G+T51P, (C) C35G+T51C, (D) H48N+T51A, and (E) T40A+H45G. Code numbers correspond to (1) ATP-dependent pyrophosphate exchange in saturating tyrosine, (2) tRNA charging in saturating tyrosine, (3) tyrosine-dependent pyrophosphate exchange in saturating ATP, (4) tRNA charging in saturating ATP, (5) formation of tyrosyl adenylate, k_{cat}/K_M for tyrosine, and (6) formation of tyrosyl adenylate, k_{cat}/K_M for ATP. Panel C shows δ's for double and triple mutants in the glutathione reductase system. Code letters correspond to mutants (A) A179G+R198M, (B) A179G+R204L, (C) R198M+R204L, (D) A179G+(R179M,R204L), (E) R198M+(A179G,-R204L), (F) R204L+(A179G,R198M), and (G) R179G+R198M+R204L. Code numbers refer to the reduction of glutathione by either (1) NADH or (2) NADPH. All data in this figure are from the secondary source (Wells, 1990).

quantity by which a component mutant will alter function; it is based on knowing that multiple mutant effects will accumulate and knowing whether their cumulative effects will be antagonistic or synergistic (Wells, 1990). At present, some of the most promising computational approaches to understanding protein structure and function, including algorithms for electrostatic calculations and potential energy minimization, generally assume that long-range pairwise interactions are additive. In order to fully understand the relationship between structure and function in proteins, however, and in order to proceed to more subtle control of protein engineering, a more exact quantitative understanding of all of the energetic interactions within a protein will be required.

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