Relationships between Apparent Binding Energies Measured in Site-Directed Mutagenesis Experiments and Energetics of Binding and Catalysis†

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ABSTRACT: The use of binding energy in molecular recognition and enzyme catalysis is currently being probed by experiments on engineered proteins. The interaction energy of an individual side chain with a substrate may be quantified by comparing the binding and rate constants for wild-type enzyme with those for a mutant in which the side chain has been truncated. An apparent binding energy ΔG_{app} is obtained. The physical significance of ΔG_{app} is analyzed with particular reference to hydrogen bonding where one partner in the bond is deleted by mutagenesis. The following conclusions have been drawn for situations where mutagenesis does not unduly perturb the structure of the protein. ΔG_{app} is always a measurement of specificity of binding and catalysis. But, it does not generally measure the incremental binding energy of the hydrogen bond ΔG_{bind}. The discrepancy between ΔG_{app} and ΔG_{bind} is especially large when mutation leaves a charged donor or acceptor unpaired. Here, ΔG_{app} overestimates ΔG_{bind} by possibly several kilocalories per mole. On the other hand, changes in ΔG_{app} ($\Delta\Delta G_{app}$) as a reaction proceeds through its intermediates and transition states are particularly amenable to simple analysis. It is shown that $\Delta\Delta G_{app}$ can measure changes in ΔG_{bind} ($\Delta\Delta G_{bind}$). For example, if there is a change in the energy of an individual bond on going from one state to the next, then $\Delta\Delta G_{app} = \Delta\Delta G_{bind}$. This rule breaks down, however, when the particular step analyzed involves the relevant groups on the enzyme and substrate binding to water in one state but to each other in the next state. In this situation, $\Delta\Delta G_{app}$ does not equal $\Delta\Delta G_{bind}$ and can seriously overestimate it when there is an unpaired charged donor or acceptor.

The importance of binding energy in protein–ligand interactions and enzyme catalysis is now increasingly being explored by site-directed mutagenesis experiments. In particular, the dissection of the structure and activity of the tyrosyl-tRNA synthetase has relied heavily on the measurement of apparent binding energies, which have detected changes in individual interaction energies as the reaction proceeds. The interpretation of the experimental data is, however, not straightforward. It is the purpose of this paper to define the various binding energy terms, to discuss what physical meaning may be attached to experimental data, and to set a rigorous framework for their analysis. The hydrogen bond is the principle interaction analyzed.

Definitions and Basic Equations

Suppose a group Y on a substrate (S) binds to a group X on an enzyme (E). Binding of X and Y is an exchange reaction in which X and Y exchange their interactions with water (w) to interact with each other (eq 1). Similarly, the water

$$\text{E–X·w} + \text{w·Y–S} \rightleftarrows \text{E–X·Y–S} + \text{ww} \qquad (1)$$

solvating X and Y is released to interact with bulk water. The binding energy of X and Y, ΔG_{bind}, may be viewed as a free energy of transfer of X and Y from water to their binding environment. ΔG_{bind} is equivalent to an incremental binding energy in the ES complex. The overall energetics of the interaction depend on the relative individual interaction energies in eq 1 and any changes in entropy and energetics of solvent structure that occur. If the interaction energy of X and w is $G_{X\cdot W}$, of Y and W is $G_{Y\cdot W}$, and of X and Y is $G_{X\cdot Y}$ and the free energy of water entering bulk solvent and any associated changes in the free energy of the solvent is G_W, then

$$\Delta G_{bind} = G_{X\cdot Y} + G_W - G_{X\cdot W} - G_{Y\cdot W} \qquad (2)$$

The value of ΔG_{bind} depends on the fit between X and Y. There is thus a spectrum of values. When there is perfect complementarity between the structures of X and Y and there is no strain or undue loss of entropy on binding, ΔG_{bind} tends to its maximum value, termed the intrinsic binding energy (Jencks, 1981). The value of ΔG_{bind} can also vary throughout a reaction, and changes in ΔG_{bind} ($\Delta\Delta G_{bind}$) are an important component of the energetics of catalysis.

Effects on Binding Energy of Modification of the Group X on the Enzyme. The group X may be modified by site-directed mutagenesis to alter the interactions with Y in two extreme ways (Figure 1, E mutated to E′). First, X may be substituted by another group which can interact with the substrate either favorably or unfavorably. This situation is too difficult to analyze in a general manner as it depends upon the specific substitutions and so will not be pursued. The second is to remove X by deletion, preferably replacing it by a hydrogen atom, so that the interaction between the the group Y and X is simply removed. Deletion can, however, have two extreme results. The first is to leave an empty cavity between the enzyme and substrate. The second is to allow free access of water to the group Y on the substrate and to the mutated region of the enzyme. The two types of deletion have different consequences, which are analyzed below.

We can measure the dissociation constants of the ES and ES′ complexes (K_S and K'_S, respectively) and use these to define the *apparent binding energy* of –XH with the substrate ΔG_{app} (Wells & Fersht, 1986):

$$\Delta G_{app} = RT \ln (K_S/K'_S) \qquad (3)$$

ΔG_{app} can be related in formal manner to the energies of the specific bonds in the complex which have been altered by mutation by setting up a thermodynamic cycle (Figure 2). If the free energy of each complex is denoted by G, then

$$\Delta G_{app} = (G_{E'} - G_E) - (G_{E'S} - G_{ES}) \qquad (4)$$

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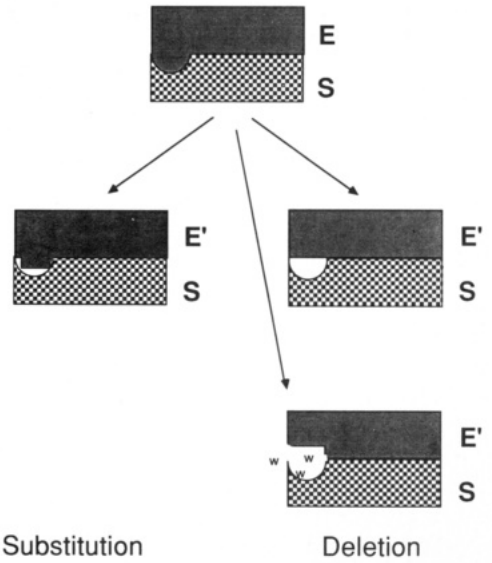

FIGURE 1: Consequences of mutating an enzyme E to E′. (Left) Substitution of a side chain for another that can still interact with the substrate leads to unknown interaction energies. (Right) Deletion of a side chain may leave an empty cavity in the enzyme and deprive the substrate of interactions with water and enzyme. Alternatively, free access of water to the cavity loses approximately the incremental binding energy of the substrate with the mutated group, dependent upon how closely the water in the cavity resembles bulk water.

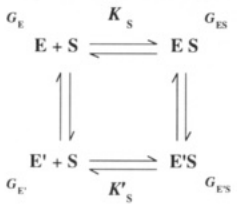

FIGURE 2: Thermodynamic cycle relating differences in dissociation constants to differences in free energies of wild-type and mutant enzymes and their enzyme–substrate complexes.

It is seen is seen that ΔG_{app} depends upon the differences in free energy between ES and E′S on the one hand and E and E′ on the other, that is the differences in structure between wild-type and mutant enzymes. Most energy terms cancel out, including those from the covalent modification to the enzyme on mutation. ΔG_{app} thus results from the dissociation energies of the specific bonds between enzyme and substrate that are changed and the energetic changes associated with any structural reorganization of the solvent and enzyme on mutation.

Relationship between ΔG_{app} and ΔG_{bind}. There is a fundamental difference between ΔG_{app} and ΔG_{bind} which is apparent from consideration of the physical processes defining each. ΔG_{bind} relates the binding energy of E and Y with each other compared with their binding energies to water. ΔG_{app} compares the binding energy of Y and E with that of Y and E′. In general, therefore, ΔG_{app} does not equal ΔG_{bind}. The physical meaning of ΔG_{app} is seen from eq 3 to be an experimental measurement of *specificity* of binding since it is derived from relative binding constants.[1]

[1] It is usually more reliable when substrates reacting with enzymes are being analyzed to use an alternative equation from kinetic determinations: $\Delta G_{app} = RT \ln [(k_{cat}/K_M)'/(k_{cat}/K_M)]$, where k_{cat}/K_M and $(k_{cat}/K_M)'$ are the specificity constants for the reactions of E and E′ with S determined from the Michaelis–Menten equation. This avoids complications from nonproductive binding and other phenomena. Since kinetic specificity is defined by the ratio $(k_{cat}/K_M)'/(k_{cat}/K_M)$ (Fersht, 1985), ΔG_{app} is clearly seen to be a direct measure of specificity.

ΔG_{app} is more a measure of ΔG_{bind} for the special case in Figure 1 where deletion allows free access of water to the group Y on the substrate and to the mutated enzyme. This is because mutation removes the interaction between X and Y but allows Y to make its solution interactions; that is, there is no transfer of Y from water when binding to E′. But, even in this example, ΔG_{app} is at best a crude measure of ΔG_{bind} because the properties of water immediately surrounding the enzymes differ somewhat from those of bulk water.

The rest of the analysis concerns ΔG_{app} for deletion mutations where hydrogen bonds are left unpaired. The analysis may be applied to modified substrates binding to a single enzyme by interchanging the symbols E and S where necessary in the equations.

Consequences of Deletion of a Group from a Hydrogen Bond To Leave an Unpaired Donor or Acceptor

Suppose a group –XH in an enzyme interacts with a substrate to form a hydrogen bond (eq 5). The reaction in water

$$S\text{–}B\cdots HOH + H_2O\cdots HX\text{–}E \rightleftarrows [S\text{–}B\cdots HX\text{–}E] + H_2O\cdots HOH \quad (5)$$

is an exchange reaction in which the donors and acceptors change partners. The overall energetics of the reaction depend on the relative strengths of the individual hydrogen bonds and the entropy changes that occur. Each individual hydrogen bond is characterized by a *hydrogen-bond dissociation energy* which is the depth of the potential energy well of that bond. Representative calculated values of hydrogen bond dissociation energies in vacuo are as follows: $HOH\cdots OH_2$, –6.4 kcal/mol; $H_2O\cdots HSCH_3$, –3.2 kcal/mol; $HOH\cdots S(H)CH_3$, –3.1 kcal/mol; imidazolium/water, –14 kcal/mol, $CH_3CO_2^-\cdots HOH$, –19 kcal/mol (Weiner et al., 1984). Charged groups have greater hydrogen-bond dissociation energies because of the higher electrostatic energies.

ΔG_{bind} is related to the hydrogen-bond dissociation energies by

$$\Delta G_{bind} = G_{EXH\cdot BS} + G_{WW} - G_{EXH\cdot W} - G_{SB\cdot W} + \Delta G_R \quad (6)$$

where $G_{EXH\cdot BS}$ is the hydrogen-bond dissociation energy of E–XH···B–S, G_{WW} is that of $H_2O\cdots HOH$, etc. ΔG_R is an energy term that contains any entropic or other energetic changes that accompany the reaction, for example, the favorable entropy change accompanying the release of bound water.

Suppose –XH is deleted by mutagenesis to give a mutant E′ so that binding is as in eq 7. The mutation is designed such

$$S\text{–}B\cdots HOH + H_2O/E' \rightleftarrows [S\text{–}B/E'] + H_2O\cdots HOH \quad (7)$$

that it removes the interaction and does not introduce any steric or other unfavorable or complicating interactions but does not allow access of water to solvate B. To allow for the possibility of the mutation causing energetic changes because of reorganization of the solvent shell of the enzyme or local structure in the enzyme, we add an additional term, ΔG_{reorg}, the "reorganization energy". This contains all the spurious factors arising from the rearrangement of the enzyme and solvent, including any perturbations of the binding of the rest of S–B to the enzyme.

Then, from eq 4

$$\Delta G_{app} = (G_{E'/W} - G_{EXH\cdot W}) - (G_{E'/BS} - G_{EXH\cdot BS}) + \Delta G_{reorg} \quad (8)$$

where $G_{E'/BS}$ is the dissociation energy of the new interaction in the mutant enzyme–substrate complex and $G_{E'/W}$ is the new

bond energy between E′ and water. The magnitude of ΔG_{reorg} is unknown and depends on the precise mutations being made. It is the experience of this laboratory from making many series of mutations at many loci that ΔG_{reorg} is probably less than 0.5 kcal/mol for mutations that appear from molecular graphics unlikely to cause gross structural artifacts.

Relationship between ΔG_{app} and ΔG_{bind} for Hydrogen Bonding. $G_{EXH\cdot BS}$, the hydrogen-bond dissociation energy of E–XH···B–S, is common to eq 6 and 8 and may be eliminated on comparing the two to give

$$\Delta G_{app} = \Delta G_{bind} - G_{WW} + G_{SB\cdot W} - G_{E'/BS} + G_{E'/W} + \Delta G_{reorg} - \Delta G_R \quad (9)$$

That is, $\Delta G_{app} \neq \Delta G_{bind}$ unless the components of the term $(-G_{WW} + G_{SB\cdot W} - G_{E'/BS} + G_{E'/W} + \Delta G_{reorg} - \Delta G_R)$ cancel out.

Experimental Evidence on Hydrogen-Bond Energetics

(*a*) *Deletion of Hydrogen-Bonding Groups Which Pair with an Uncharged Donor or Acceptor.* A recent compilation of measurements of uncharged hydrogen bonds in enzyme–ligand and nucleotide–nucleotide complexes reveals that both ΔG_{app} and ΔG_{bind} are in the range 0.5–1.8 kcal/mol for bonds where there are no unfavorable steric factors (Fersht et al., 1985; Fersht, 1987). The similarity between ΔG_{app} and ΔG_{bind} may arise for two reasons. First, the term $(-G_{WW} + G_{SB\cdot W} - G_{E'/BS} + G_{E'/W} + \Delta G_{reorg} - \Delta G_R)$ in eq 9 may cancel out, and rough calculations suggest that this is so. Second, some of the mutations may allow access of water to the remaining donor or acceptor. As explained earlier, ΔG_{app} roughly approximates to ΔG_{bind} under these circumstances. Nevertheless, the similarity between ΔG_{app} and ΔG_{bind} in these cases must be regarded as fortuitous.

(*b*) *Deletion of Hydrogen-Bonding Groups Which Pair with a Charged Donor or Acceptor.* Deletion of a bond to a charged donor/acceptor weakens binding by a much greater amount, some 3–6 kcal/mol for either one or both partners being charged (Lowe et al., 1987). ΔG_{app} may be related to ΔG_{bind} by substituting the hydrogen-bond dissociation energy for a charged bond into eq 9, for example, that for SB^-···HOH ($G_{SB^-\cdot W}$), to give eq 10. $G_{SB^-\cdot W}$ is by far the most dominant

$$\Delta G_{app} = \Delta G_{bind} - G_{WW} + G_{SB^-\cdot W} + G_{E'/BS} - G_{E'/W} + \Delta G_{reorg} - \Delta G_R \quad (10)$$

component in the term $(-G_{WW} + G_{SB^-\cdot W} + G_{E'/BS} - G_{E'/W} + \Delta G_{reorg} - \Delta G_R)$. ΔG_{app} thus considerably overestimates ΔG_{bind}. Because $G_{SB^-\cdot W}$ is so high, it is likely that there will be compensating interactions of SB^- with water or the protein. In other words, ΔG_{reorg} will be high, and so eq 10 becomes difficult to interpret.

Difference Energy Diagrams and Relationship of $\Delta\Delta G_{app}$ to $\Delta\Delta G_{bind}$

Understanding enzyme catalysis requires knowing the interaction energies between the enzyme and substrate throughout the whole course of reaction. Differential binding in reactions of the tyrosyl-tRNA synthetase has been shown by determining ΔG_{app} for the successive intermediates on the reaction pathway (Wells & Fersht, 1985, 1986; Ho & Fersht, 1986; Fersht et al., 1986). Differences in ΔG_{app} along a reaction pathway, $\Delta\Delta G_{app}$, can be readily interpreted by the following analysis. Suppose there is a bond –XH···B– between EXH and BS and it changes in strength throughout the reaction as it proceeds through the various intermediate and transition-state complexes. We can apply eq 8 to each state. The terms $G_{E'/W}$ and $G_{EXH\cdot W}$ are constants in eq 8 for a particular mutation and so their differential with respect to reaction coordinate is zero. If ΔG_{reorg} and $G_{E'/BS}$ are also constant along the reaction pathway (i.e., $\Delta\Delta G_{reorg} = 0$ when the structural changes in the solvent and enzyme on mutation do not change during the course of reaction, which are reasonable assumptions for many situations), then

$$\Delta\Delta G_{app} = \Delta G_{EXH\cdots S} \quad (11)$$

Or, directly from eq 9 with the same assumption that $\Delta\Delta G_{reorg} = 0$

$$\Delta\Delta G_{app} = \Delta\Delta G_{bind} \quad (12)$$

That is, $\Delta\Delta G_{app}$ is a direct measure of the changes in bond dissociation energy of –XH···S as it changes as the reaction proceeds through intermediate steps. Thus, site-directed mutagenesis does measure the subtle changes in bond energies as reactions proceed.

An Exception Where $\Delta\Delta G_{app} \neq \Delta\Delta G_{bind}$. The rule that $\Delta\Delta G_{app} = \Delta\Delta G_{bind}$ when $\Delta\Delta G_{reorg} = 0$ breaks down when the bond –XH···B– between EXH and BS does not exist at all in one of the enzyme–bound complexes but is formed in a subsequent complex. This could occur when a step in the reaction is accompanied by an isomerization in an ES complex which involves groups on E and S losing bonds to water and forming an intramolecular hydrogen bond as an integral step, as in eq 13. This is now equivalent to the energetic changes as in eq 5 and so the analysis that applied to ΔG_{app} in eq 9 applies to $\Delta\Delta G_{app}$ (eq 14). For example, just as $\Delta\Delta G_{app}$ seriously overestimates ΔG_{bind} when a donor to or an acceptor of a charged group is mutated, so $\Delta\Delta G_{app}$ overestimates $\Delta\Delta G_{bind}$. For subsequent steps following the isomerization in eq 13, the relationship $\Delta\Delta G_{app} = \Delta\Delta G_{bind}$ should again hold.

$$E\text{–}XH\cdots OH_2\cdot S\text{–}B\cdots HOH \rightleftarrows [E\text{–}XH\cdots B\text{–}S] + HOH\cdots OH_2 \quad (13)$$

$$\Delta\Delta G_{app} = \Delta\Delta G_{bind} - G_{WW} + G_{SB\cdot W} - G_{E'/BS} + G_{E'/W} + \Delta G_{reorg} - \Delta G_R \quad (14)$$

In general, however, $\Delta\Delta G_{app}$ is a more reliable quantity than ΔG_{app} for two reasons. First, the unknown quantity on mutagenesis is the value of ΔG_{reorg}, the sum of any of the free energy perturbations in the enzyme and solvent on mutation due to local variation in structure. Although it is likely that ΔG_{reorg} may not be negligible in some cases, ΔG_{reorg} is likely in many cases to alter the energy level of unligated enzyme and all the complexes by the same amount so that $\Delta\Delta G_{reorg}$ is very small. Second, $\Delta\Delta G_{app}$ may be measured with high precision because values are calculated from the ratios of first-order rate constants for the interconversion of enzyme-bound species at saturating concentrations of substrates (Wells & Fersht, 1986). These rate constants are, therefore, independent of both the concentration of enzyme and the concentration of substrate and hence problems of purity, active site titer, and dispensing errors.

Illustrative Examples with Tyrosyl-tRNA Synthetase

The tyrosyl-tRNA synthetase catalyzes the formation of enzyme-bound tyrosyl adenylate from tyrosine and ATP (eq 15). Several side chains at the active site have been mutated

$$E \xrightleftharpoons{Tyr} E\cdot Tyr \xrightleftharpoons{ATP} E\cdot Tyr\cdot ATP \rightleftarrows E\cdot Tyr\text{–}AMP\cdot PP_i \rightleftarrows E\cdot Tyr\text{–}AMP + PP_i \quad (15)$$

to remove hydrogen-bond donating groups, a selection of data being given in Table I. Application of the above theoretical

Table I: Values of ΔG_{app} for Reactions of Tyrosyl-tRNA Synthetase[a]

complex	ΔG_{app} (kcal/mol)				
	Tyr–Phe-34	Cys–Gly-35	His–Gly-48	His–Ala-40	His–Ala-45
E·Tyr	−0.52	0.05	−0.39	0.05	−0.56
E·Tyr·ATP	−0.49	0.08	−0.83	0.17	−0.61
E·[Tyr–ATP]*	−0.53	−1.26	−1.63	−5.05	−4.11
E·Tyr–AMP·PP_i	−0.69	−1.66	−1.62	<−3	<−3
E·Tyr–AMP	−0.88	−1.64	−1.85	0.23	−0.29

[a] Data from Wells and Fersht (1986) and R. J. Leatherbarrow and A. R. Fersht (unpublished results). E·[Tyr–ATP]* is the transition state for the reaction.

work enables a deeper analysis of the data than previously given.

There is detailed knowledge about the interactions of Tyr with the enzyme as the crystal structure of the E·Tyr complex has been solved (Brick & Blow, 1987). The structure of the E·ATP complex is as yet unsolved, and the interactions of ATP with the enzyme have been inferred from the crystal structure of the E·Tyr–AMP complex.

Removal of the hydroxyl group of Tyr-34 of the enzyme to give Phe-34 deletes a hydrogen-bond donor to the tyrosine hydroxyl of the substrate. This is reflected in a destabilization of the E·Tyr complex of about 0.5 kcal/mol. Whether or not the value of ΔG_{app} is equal to ΔG_{bind} depends on whether or not there is a water molecule in the cavity in the mutant enzyme which is hydrogen bonded to the substrate tyrosine hydroxyl and how similar the energetics of binding of this water molecule are to those of bulk water. (X-ray crystallographic studies are in progress on the mutant enzyme–tyrosine complex to search for the water.) The subsequent increases in ΔG_{app}, $\Delta\Delta G_{app}$, do give the increases in the hydrogen-bond strength, $\Delta\Delta G_{bind}$, and show that there is increased stabilization of the E·Tyr–AMP complex.

Mutation of Cys → Gly-35 removes a donor from the enzyme to the 3′-OH of the ribose of Tyr–AMP. ΔG_{app} is approximately zero in the E·Tyr·ATP complex, rising to 1.26 kcal/mol in the transition state. As there is as yet no kinetic or direct structural evidence for hydrogen bonding from Cys-35 to the ribose hydroxyl in the E·Tyr·ATP complex, there is the possibility that the change from E·Tyr·ATP to the transition state E·[Tyr–ATP]* is an example of eq 13. That is, ATP may bind first without forming a hydrogen bond to Cys-35 (both remaining bonded to water), and there could be a subsequent conformational change to form the bond. If so, the value of $\Delta\Delta G_{app}$ is not necessarily equal to the value of $\Delta\Delta G_{bind}$ for this process. However, the hydrogen bond is clearly present in the transition state, and so the subsequent increases to 1.65 kcal/mol in the E·Tyr–AMP·PPi and E·Tyr–AMP complexes do measure the increases in $\Delta\Delta G_{bind}$. His-48 interacts with the nucleotide throughout the reaction, and so the increases in $\Delta\Delta G_{app}$ again measure the increases in $\Delta\Delta G_{bind}$.

There is a binding site for the γ-phosphate or ATP between Thr-40 and His-45 which binds only in the transition state of the reaction (Leatherbarrow et al., 1985). As there is no evidence from the energetics in Table I that hydrogen bonds are present in the E·Tyr·ATP complex, it is possible that this is again an example of eq 13. Indeed, it was suggested that the γ-phosphate swings into the binding site as the transition state is reached, sloughing off hydrogen-bonded water (Leatherbarrow et al., 1985). If so, the value of $\Delta\Delta G_{app}$ for E·Tyr·ATP going to E·[Tyr–ATP]* will overestimate the true $\Delta\Delta G_{bind}$, as described above for eq 10 and 14, and so overestimate the extent of transition-state stabilization. The high values of 4–5 kcal/mol support this view. The same reasoning applies to the high values of $\Delta\Delta G_{app}$ observed in the following paper (Fersht et al., 1988). The mutagenesis experiments thus pinpoint crucial charged residues but may overestimate the stabilization energies.

Conclusions

ΔG_{app} is always an experimental measurement of specificity of binding, and so the experimental values for ΔG_{app} may be generally applied to calculating specificities of binding. ΔG_{app} does not in general equal ΔG_{bind} and is at best only a crude approximation of ΔG_{bind} in circumstances where mutation allows access of water to the region where deletion occurs. In particular, ΔG_{app} measured for mutation of partners of charged group can seriously overestimate ΔG_{bind}. Values of $\Delta\Delta G_{app}$ from differences energy diagrams do frequently measure true changes in binding energies as reactions proceed.

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