Evaluating Electrostatic Contributions to Binding with the Use of Protein Charge Ladders

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have been described in many cell types (21). It is likely that type I myosins play an important role in endocytic internalization via these pathways.

REFERENCES AND NOTES

9. M. I. Gell and H. Riezman, data not shown.
10. MYO3 and MYO5 are deleted in the same diploid to obtain a targeted deletion, strain RH3375 (MATa\MATa ade2\ade2 his3\his3 myo5\Delta::HIS3/MYO3 myo5\Delta::TRP1/MYO5 leu2\leu2 lys2\lys2 trp1\trp1 caraU3a bar1\bar1). From this strain the RH3375, RH3377, and RH3378 strains were generated by tetrad dissection (Table 1). Deletion of MYO3 has been described (6). For deletion of MYO5, the fragments Eco RI-Hind III and Pst1-Bgl II (Fig. 1A) were used to flank a 0.9-kb DNA fragment carrying the TRP1 gene. The spores were analyzed by replica plating on synthetic dextrose medium containing the corresponding amino acids. None of the predicted double mutants (TRP1::HIS3) out of 20 dissected tetrads formed colonies. A double mutant harboring plasmid pmyo5-1 or pMYO5 (13) was able to form pinpoint-sized colonies upon plasmid loss. When the pmyo5-1-carrying strain was shifted to 37°C, the proportion of un budding cells did not change.
13. E511 (Glu472) of the putative actin binding domain (C. G. Johnston, J. A. Pendergast, R. A. Singer, J. Cell Biol. 113, 539 (1991)) corresponds to E472 (Glu472) in Myo6p (Fig. 1A). The allele E472K (Glu472Lys472) (myo5-1) was generated by PCR and cloned into a centromere-based plasmid YCplac33; Gene 74, 527 (1988) to generate pmyo5-1. Wild-type MYO5 was also cloned into YCplac33 to generate pMYO5.
14. RH3380, RH3383, and RH3382, and RH3384 strains (Table 1) were generated by tetrad dissection from RH3375 (6) transformed with pmyo5-1 or pMYO5 (12). RH3382 and RH3384 were used as controls for strains RH3380 and RH3383 in order to guarantee similar amounts of expression of WT and ts MYO5p. Strains RH3382 and RH3384 behaved in all experiments exactly as did the RH3375 strain, except for α-factor uptake at 37°C, where the initial uptake rates were reduced by approximately 25%.
23. We thank Riezman laboratory members for discussions; S. Schröder, A. L. Munn, A. Wesp, C. Söllterlin, and M. Schönholzer for critical reading of the manuscript; T. Aust and N. Stern for technical assistance; and H. Goodson for the plasmid to delete MYO3. Funded by a grant from the Swiss National Science Foundation (to H.R.), M.I.G. was supported by a European Molecular Biology Organization long-term fellowship.
19 December 1995; accepted 12 February 1996

Evaluating Electrostatic Contributions to Binding with the Use of Protein Charge Ladders

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Electrostatic interactions between charges on ligands and charges on proteins that are remote from the binding interface can influence the free energy of binding (ΔG一审). The binding affinities between charged ligands and the members of a charge ladder of bovine carbonyl anhydrase (CAII) constructed by random acetylation of the amino groups on its surface were measured by affinity capillary electrophoresis (ACE). The values of ΔG一审 derived from uncharged ligands are approximately linearly with the charge. Opposite charges on the ligand and the members of the charge ladder of CAII were stabilizing; when charges were destabilizing. The combination of ACE and protein charge ladders provides a tool for quantitatively examining the contributions of electrostatics to free energies of molecular recognition in biology.

Although charged groups appear in a majority of biological molecules, and electrostatic interactions between these groups undeniably contribute energetically to many important biological interactions, it has been difficult to evaluate these contributions quantitatively. A recent, stimulating review of the influence of electrostatic interactions in biochemistry by Honig (1) analyzed this subject in detail and drew a number of startling inferences; for example, in some circumstances (2, 3), interactions between opposite charges may be destabilizing, rather than stabilizing as expected for idealized electrostatic interactions in vacuum (4).

Efforts to quantify electrostatic effects in interactions of proteins with ligands have centered on proteins modified by site-specific mutagenesis (5). This technique, although powerful, is labor-intensive and is cumbersome when used to generate proteins that are multiple mutated. Here we summarize the energetics of interaction of the members of a protein charge ladder (6) derived from bovine carbonyl anhydrase II (CAII) (E.C. 4.2.1.1, containing two isozymes of isoelectric points 5.4 and 5.9, respectively) (7) with benzzenesulfonylmides substituted in the para position with charged and neutral groups. CAII is a roughly spherical Zn(II) metalloenzyme with a conical binding pocket. This pocket is lined with both hydrophobic and polar residues but not with charged residues (7). The combination of affinity capillary electrophoresis (ACE) (8) and charge ladders derived from CAII and other proteins constitutes a versatile and convenient system with which to define electrostatic contributions to the energetics of the association of charged proteins and charged ligands.

Treatment of CAII with acetic anhydride generates a set of proteins in which distributions of positively charged Lys ε-ammonium groups are converted to neutral N-acetyl derivatives (Eq. 1). These sets of modified proteins appear in capillary electrophoresis (CE) as a set of evenly spaced peaks, which we call a "protein charge ladder" (6). In Eq. 1, n is the number of acetylated amines [CAII has 18 Lys ε-NH₂⁺, 26 Asp or Glu-Co²⁻, and 9 Arg-NH₂⁺(NH₃⁺)²⁻ groups (7)] and Z₀ and Zₙ are the charges of the native protein and proteins having n modified Lys groups, respectively. In CE, the electrophoretic mobility (µₚ) of a protein is proportional to its charge and inversely correlated with its molecular weight (M)

$$ \mu_p = \frac{C_p}{M^2} Z_n = \frac{C_p}{M^2} Z_0 - \frac{C_p}{M^2} \text{n} \quad (2) $$

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where $C_n$ is a proportionality constant that includes the influence of screening of charge by counterions in solution, and $a = 3/7$ for globular proteins (9, 10). In the case of CALI, the families of acetylated derivatives differ in charge by integral units; they differ in molecular weight only minimally (the molecular weight of an acetyl group is 42 daltons; that of CALI is 30 kD). Therefore, all proteins with the same value of $n$ have essentially the same electrophoretic mobility. ACE measures the changes in the mobility of the proteins in the charge ladder as a function of the concentration of a ligand in the electrophoresis buffer and yields the binding constant to each member of the charge ladder simultaneously (8).

We have determined binding constants for each member of the charge ladder of CALI (11) with seven structurally related ligands differing in charge and position of charge relative to the sulfonamide group (12) (Fig. 1). Scatchard analysis of the changes in electrophoretic mobility of each member of the CALI charge ladder with concentration of ligand yields its value of the binding constant $K_b$ (8). Analysis of these data gives free energies of binding ($\Delta G_0$) (Fig. 2).

We draw three conclusions from these data. First, acetylation of the Lys e-amino groups of CALI does not influence its binding to neutral ligands (13): all members of the charge ladder bound ligands 2, 3, and 7 equally. This lack of discrimination suggests that the acetylated derivatives of CALI retain the native conformation at the active site, even when 16 $\text{NH}_3^+$ groups are converted to $\text{NHAc}$ groups. Second, binding constants of the members of the charge ladder depend on the charge on the protein and the charge on the ligand in a regular way (14): the more negatively charged members of the charge ladder bound less tightly to a negatively charged ligand (4) and more tightly to a positively charged ligand (1) than did the less negatively charged members. Quantitatively, the magnitude of the dependence of $\Delta G_0$ on the net charge of CALI and its derivatives was 0.05$Z_n$ to 0.12$Z_n$ kcal $\text{mol}^{-1}$ for the charged ligands 1 and 4 (15). Third, to a first approximation, the position of acetylation has little influence on the values of binding constants: the mobilities of all derivatives of CALI having the same overall charge (a single peak in the charge ladder) seemed to shift together as the concentration of the ligand increased. We believe that the small broadening of peaks in the center of the charge ladder ($n = 6$ to 12) reflects a slight heterogeneity in binding affinity within families of acetylated derivatives of CALI having the same net charge.

The addition of one unit of negative charge to CALI stabilizes (or destabilizes) its interaction with ligand 1 (or 4) by 0.05 to 0.1 kcal $\text{mol}^{-1}$ (Fig. 2A). A simple, approximate, Coulombic model indicates that this value is physically reasonable. We model CALI as a spherical solid of radius $r_o = 20$ Å (16) with a charge $Z_n = Z_o - n$ ($Z_o = 3.5$ at pH 8.3 (17)) distributed uniformly over its surface. We assume that the ionic strength of the solution is zero and that the dielectric constant outside the sphere is that of water ($\epsilon = 80$); the model is independent of the value of $\epsilon$ inside the sphere. Bringing a test charge in from infinite distance (defined as being at potential energy $V = 0$) to a distance $r$ (for $r \leq r_o$) results in a change $\Delta V = Z_n/4\pi\epsilon_0 r_o^2$ $\approx 0.2Z_n$ kcal $\text{mol}^{-1}$ (10), where $\epsilon_0$ is the permittivity of free space; the observed value of 0.05$Z_n$ to 0.12$Z_n$ kcal $\text{mol}^{-1}$ is consistent with this estimate (18). We are currently performing more rigorous calculations using Poisson-Boltzmann methods (1).

This electrostatic model of CALI-ligand interaction predicts that the magnitude of

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**Fig. 1.** Electropherograms of the binding of ligand 4 to the charge ladder of CALI. Increasing concentrations of ligand 4 in a buffer of 25 mM tris and 192 mM Gly (pH = 8.3) were used as the electrophoresis buffer. The neutral marker was 4-methoxybenzyl alcohol. The number of modified e-amino groups ($n$) and net charge of the protein in the charge ladder ($Z_n = Z_o - n$) are indicated below the electropherograms. The small peaks marked with (0) are impurities in the sample. Equivalent results were obtained for the other ligands.

**Fig. 2.** Dependence of the free energy of binding ($\Delta G_0$) on the charge of CALI ($Z_n$) in the charge ladder and on (A) the charge on the ligands (numbers in parentheses following the ligands indicate charge) and (B) the location of the charge relative to the sulfonamide group. The binding affinity of each member of the charge ladder to ligands 1 through 7 was measured by ACE in a buffer of 25 mM tris and 192 mM Gly (pH = 8.3). Because of slight broadening of the peaks near the center of the charge ladder ($n = 6$ to 12), the uncertainties in the values of $\Delta G_0$ for these derivatives are larger than those for the other parts of the charge ladder (for some values of $n$, the peak broadening required missing data). The slopes ($\Delta G_0/\Delta Z$) from the linear regression analysis of $\Delta G_0$ versus $Z_n$ yielded the magnitudes of influence of charges on CALI-ligand interactions. Values of $\Delta G_0/\Delta Z$ (in kilocalories per mole per charge) for these ligands: 1, 0.05 ± 0.01; 2, 0.01 ± 0.01; 3, 0 ± 0.01; 4, 0.10 ± 0.01; 5, 0.07 ± 0.02; 6, 0.02 ± 0.02; and 7, 0 ± 0.01.
electrostatic interactions will decrease upon increase of the distance between the charged group on the ligand and the binding site of CAII. To test this hypothesis, we compared ligands 4, 5, and 6, in which the negatively charged carboxylate group was separated from the sulfonamide group by increasing numbers of bonds (Fig. 2B). The dependence of free energy of binding on charge, ΔΔGij/ΔZ, for ligands 4, 5, and 6 was $0.10 \pm 0.01, 0.07 \pm 0.02,$ and $0.02 \pm 0.02$ kcal mol$^{-1}$ charge$^{-1}$, respectively. As expected, the interactions between the charges on ligands and proteins decreased as the number of bonds between the sulfonamide group and the charged group increased. The value of ΔΔGij/ΔZ for 4 is approximately twice that of the shorter ligand 1; we have not established the origin of this difference.

Three characteristics of the combination of ACE and charge ladders are particularly useful for study of electrostatic contributions to the free energies of protein-ligand interactions. First, it generates large numbers of directly comparable data in a straightforward experimental system. Second, charge ladders can be generated from a large number of proteins, and although only certain charge ladders behave as simply as that from CAII, the technique has useful generality (9). Third, the technique readily permits quantitative evaluation of both intense (ion composition and temperature) and extensive (ionic strength and pH) influences on the electrostatic contribution to biological interactions.

REFERENCES AND NOTES

4. The observation that interactions between colloidal particles that have like charge can, in certain instances, be attractive further complicates our understanding of electrostatic effects in ionic media [N. Iso and H. Yoshida, Acc. Chem. Res. 29, 3 (1996); K. S. Schmitz, ibid., p. 7].
11. The charge ladder of CAII was prepared as follows. The pH of three solutions of native CAII (0.1 mM in distilled water, 0.5 mM) was adjusted to pH 10 by addition of 0.1 N NaOH and was maintained at this value. Acetic anhydride (10, 20, and 40 equiv, respectively, 100 mM in dimethyl sulfoxide) was added. After 30 min, the three solutions were mixed and diluted to 10 μM in a buffer of 25 mM Tris and 192 mM Gly (pH = 8.3). This procedure gave a sample in which each member of the charge ladder could be easily analyzed.

12. We used a Beckman PIAC 5500 system for CE. General conditions: operated at 15 kV; temperature 7 – 37°C; maintained by liquid cooling; capillary inner diameter, 50 μm; total length of capillary, 47 cm; length from inlet to detector, 40 cm. For the ACE experiments, different compositions of the ligand were prepared in a buffer of 25 mM Tris and 192 mM Gly (pH = 8.3). The capillary was flushed with 0.1 N NaOH, distilled water, and electrophoresis buffer for 2 min before each experiment.
13. Using competitive ACE (6), we measured the binding affinities of the neutral ligands 2 and 3 to CAII by competition with ligand 1 (+1 charge); and that of neutral ligand 7 by competition with ligand 5 (–1 charge).
14. A conventional fluorescence assay on samples containing mixtures enriched in low-Z, and high-Z fractions of the charge ladder of CAII confirmed qualitatively the influence of its charge, and that of its ligands, on ΔΔGij.
15. The lines that correlate ΔΔGij with Zc in Fig. 2 can be extrapolated to Zc = 0. At this point, the influence of charge-charge interactions on binding should disappear. For ligands with the same structure inside the active site (Fig. 2A), the lines can be extrapolated to a common point (ΔΔGij = −8.4 ± 0.1 kcal mol$^{-1}$) at Zc = 0; for ligands with different structure inside the active site (Fig. 2B), the lines do not intersect at Zc = 0, and therefore, other factors (for example, $pK_a$ values of sulfonamide groups or hydrophobic interactions) must contribute to differences in binding.
16. The dimensions of CAII taken from its crystal structure are 41 Å by 41 Å by 47 Å [A. Liljas et al., Nature 235, 131 (1972)].

Adaptive Evolution of Human Immunodeficiency Virus–Type 1 During the Natural Course of Infection

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The rate of progression to disease varies considerably among individuals infected with human immunodeficiency virus–type 1 (HIV-1). Analyses of semiannual blood samples obtained from six infected men showed that a rapid rate of CD4 T cell loss was associated with relative evolutionary stasis of the HIV-1 quasispecies virus population. More moderate rates of CD4 T cell loss correlated with genetic evolution within three of four subjects. Consistent with selection by the immune constraints of these subjects, amino acid changes were apparent within the appropriate epitopes of human leukocyte antigen class I–restricted cytotoxic T lymphocytes. Thus, the evolutionary dynamics exhibited by the HIV-1 quasispecies virus populations under natural selection are competitive with adaptive evolution.

In general, the natural history of HIV-1 infection in humans follows a defined pattern with well-characterized features (1–3); however, the rates of development of disease and the survival times in different individuals vary widely (4). The pathogenic potential of the virus (5–8) and the immunopathogenic effects of the immune response (9) have each been postulated to explain the observed differences in progression to disease. One hypothesis that might explain the variable course is that the loss of CD4 T cells in HIV–infected individuals is primarily due to increasing antigenic diversity that, beyond a threshold, exceeds the capacity of the immune response to regulate viral population growth (10).

To evaluate this hypothesis critically, we directly measured the levels of HIV–1 RNA and tracked viral sequence changes that occurred in concert with the humoral and cellular immune responses in a well-defined cohort of HIV–1–infected individuals. Six men with confirmed HIV–1