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Role of the histidine residues of visna virus nucleocapsid protein in metal ion and DNA binding

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Abstract Zinc finger (ZF) domains in retroviral nucleocapsid proteins usually contain one histidine per metal ion coordination complex (Cys-X_2-Cys-X_4-His-X_4-Cys). Visna virus nucleocapsid protein, p8, has two additional histidines (in the second of its two ZFs) that could potentially bind metal ions. Absorption spectra of cobalt-bound ZF2 peptides were altered by Cys alkylation and mutation, but not by mutation of the extra histidines. Our results show that visna p8 ZFs involve three Cys and one His in the canonical spacing in metal ion coordination, and that the two additional histidines appear to interact with nucleic acid bases in p8–DNA complexes.

Key words: Histidine residues; Visna virus nucleocapsid protein; Metal ion binding; DNA binding; Zinc finger domains

1. Introduction

Retroviral nucleocapsid (NC) proteins bind nucleic acids, package genomic RNA and are essential for retroviral replication [1,2]. These proteins typically bind zinc ions with one histidine and three cysteine residues. The pattern, a highly conserved motif, constitutes a zinc finger (ZF) of the type **Cys-X_2-Cys-X_4-His-X_4-Cys** [3–5].

Visna virus NC protein p8, which has two ZFs, differs from other retroviral NC proteins in that the second ZF motif (spanning residues 39–52) includes three histidines; two extra histidines are found between Cys-39 and Cys-42. The presence of these extra His residues and their location leads to the question: does visna virus NC protein bind zinc ions through the typical retroviral CCHC motif or are the additional histidines involved in binding metal ions?

We addressed this question by preparing synthetic, mutant ZF peptides. Using Co(II) instead of Zn(II) as the metal ligand, we were able to apply absorbance spectroscopy to determine whether the extra histidine residues were involved in metal ion chelation.

We ascertained that the extra histidines residues do not bind divalent metal ions, leading us to speculate that the hydrophobic imidazole side chains may participate in nucleic acid binding, as do similarly positioned aromatic residues in NC proteins from other retroviruses [6–8]. We present below the results of fluorescence experiments in support of this hypothesis.

2. Materials and methods

The amino acid sequence of p8, the NC protein from visna virus strain 1514 [9,10], is LAQAL^5 RPQGK^{10} AGHKG^{15} VNQK**C**^{20} YN**C**GK^{25} P**G**H**L**A^{30} R**Q**C**R**Q^{35} GII**C**H^{40} H**C**G**K**R^{45} **G**H**M**QK^{50} D**C**RQK^{55} KQQGN^{60} NRRGP^{65} RVVSA^{70} PPPML^{75} (apo mol. wt = 8319.74 Da, p*I* = 11.46; ZF regions are underlined, and putative metal ion chelating residues are in bold face). To isolate p8 in pure form, a suspension of sheep choroid plexus cells infected with visna virus was made 8 M in guanidine–HCl, 40 mM in Tris–HCl, pH 8.5, and 2% (v/v) in 2-mercaptoethanol. The suspension was mixed and allowed to sit at room temperature under nitrogen for 90 min, acidified to pH 1.2 with trifluoroacetic acid, then purified using reversed-phase HPLC as previously described [11]. Fractions containing p8 were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie brilliant blue staining, lyophilized, and rechromatographed. The amino acid composition, primary sequence, and the molecular mass measured by MALDI-TOF mass spectrometry of the pooled p8 material were in agreement with the published sequence. 2 mol of zinc acetate was added to each mol of purified p8, which was lyophilized and stored at −80°C until used.

We designed synthetic peptides (prepared by Macromolecular Resources, Ft. Collins, CO) to test histidine participation in metal ion binding. Peptides began two residues before the initial cysteine and ended two residues after the last cysteine in the ZF motif. Peptides spanning ZF1 and ZF2 were synthesized. ZF1 was made with Pro-26 replaced by Glu to prevent proline isomerization and maintain a charged side chain, usually found at this position. Peptides were purified to >90% purity by HPLC and assessed by MALDI-TOF mass spectrometry. Lyophilized peptides were resuspended in a 20% acetonitrile, 0.1% trifluoroacetic acid aqueous solution. A stoichiometric amount of metal ion (1 g atom per mol of ZF) was added to each peptide. Zinc acetate (Mallinckrodt, Paris, KY), cobalt(II) chloride, and cadmium sulfate (both from Aldrich, Milwaukee, WI) were used for metal ion reconstitution. Peptide–metal salt samples were lyophilized and stored at −80°C until used. All peptides were dissolved in 10 mM phosphate buffer, pH 7 for our experiments. In addition to the synthetic wild type peptides, the following synthetic mutant peptides were used in our study:

- $ZF2^Y$: extra His residues were replaced by Tyr (H40Y, H41Y) in this peptide. Tyrosine was chosen because it would not chelate metal ions, and its phenolic side chain is fluorescent, planar and of a size similar to histidine's imidazole ring;
- $ZF2^S$: three Cys residues were replaced by Ser (C39S, C42S, C52S) in this mutant, which was used to test whether the extra histidine residues 40 and 41 were capable of chelating divalent metal ions in the absence of cysteine thiolates;
- Double ZF: (residues 18–54) includes both ZFs in p8. Tyr-21 in the first ZF was replaced by tryptophan (Y21W) to enhance the peptide's fluorescence emission intensity and more readily assess its DNA binding capacity.

These mutant peptides were compared with the wild type forms, before and after reaction with the Cys-alkylating reagent *N*-ethylma-

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leimide (NEM, Sigma, St. Louis, MO). NEM reaction was carried out at room temperature for thirty min at a 5:1 NEM:Cys molar ratio.

Oligonucleotides used in binding affinity titrations were obtained from the Recombinant DNA Laboratory, SAIC Frederick. Polynucleotides were purchased from Pharmacia (Piscataway, NJ).

Absorbance spectra were measured with a Beckman (Columbia, MD) DU 640 spectrophotometer. Fluorescence spectra were measured with a Shimadzu (Columbia, MD) RF-5301 PC spectrophotofluorometer. DNA binding was assessed qualitatively by adding $d(I)_8$ to p8 sequences and recording either emission spectra (excitation at 274 nm for p8 and 280 nm for the double ZF peptide) or fluorescence intensity at the wavelength of maximum emission. Fluorescence titrations were based on the intrinsic fluorescence of visna p8. The technique has been described previously [7,8,12,13].

3. Results and discussion

3.1. ZF1 and ZF2 absorbance spectra

To assess the composition and geometry of the divalent metal ion chelation sphere in visna ZFs, we used Co(II) in place of Zn(II). Co(II) complexes absorb visible light; their optical absorbance, line shape and position vary with the number of N and S ligands bound by Co^{2+} [14–16].

Cobalt-bound ZF1 and ZF2 peptides had similar absorbance spectra (Fig. 1). Each peptide showed a charge transfer peak at 309 nm; a *d-d* transition peak with two shoulders was seen at 550–750 nm in the pattern characteristic of one N and three S ligands bound to Co^{2+} [14–16]. The peak's line shape denotes an unsymmetrical disposition of ligand groups with tetrahedral geometry. The spectral similarity between the two ZF peptides strongly suggests that their coordination sphere is identical. Since ZF1 contains only three cysteine and a single histidine residue, this implies that all three cysteines in ZF2 are involved in metal ion binding. Additional evidence was gathered using the mutant ZF sequences (see below).

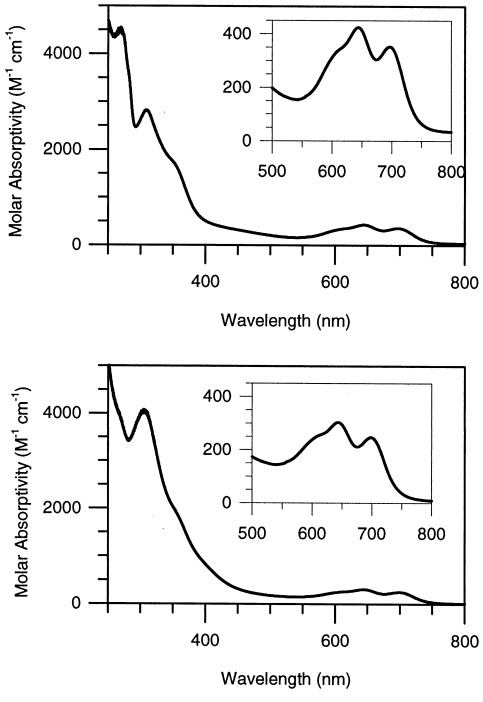

Fig. 1. Absorbance spectrum of Co(II) complexes of ZF1 (top panel) and ZF2 (bottom panel). Insets show *d-d* transition peaks. Absorption of Tyr at 275 nm was detected in ZF1. ZF solutions were 150 μM in 10 mM sodium phosphate, pH 7, and were placed in 1 cm pathlength quartz micro-cuvettes.

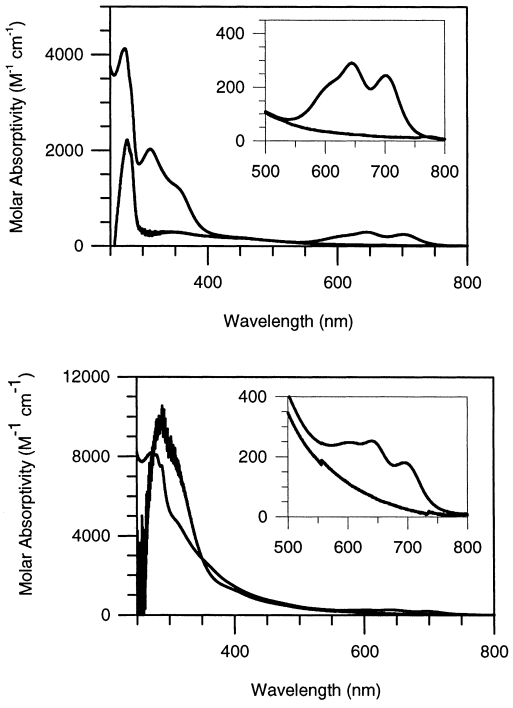

Fig. 2. Absorbance spectrum of $ZF2^Y$ (top panel) and double ZF peptide (bottom panel) complexed with Co(II) before and after Cys alkylation with NEM, upper and lower traces, respectively. Inset shows *d-d* transition peaks. Conditions were as described above.

3.2. $ZF2^Y$ absorbance

Substitution of tyrosine for histidine in the p8 ZF2 peptide did not alter its absorbance peak wavelength in the near-UV and visible regions of the spectrum, although absorbance from tyrosine (which replaced histidine at positions 40 and 41) was apparent in the far-UV (Fig. 2). $ZF2^Y$ had charge transfer and *d-d* transition peaks in the same position as seen in ZF1 and ZF2, albeit the molar absorptivity of the peaks at 500–800 nm was somewhat lower (Fig. 2). These findings demonstrate that His-40 and His-41 are not essential for Co(II) binding.

Consequently, we would expect cysteine alkylation to prevent metal ion coordination. Indeed, $ZF2^Y$ treated with NEM showed no charge transfer or *d-d* transition peaks (Fig. 2). Thus, at least one cysteine thiolate in the ZF2 peptide is required for Co(II) binding.

3.3. $ZF2^S$ absorbance

Testing whether His residues at non-canonical positions may participate in metal ion chelation in the absence of cysteine residues, we studied $ZF2^S$, a peptide in which cysteine was replaced by serine. No charge transfer or *d-d* peaks were seen in $ZF2^S$ solutions in the presence of Co(II) ions (not shown). This is in agreement with our results for NEM-reacted $ZF2^Y$.

It has been reported, however, that the binding affinity of ZF sequences for Co(II) may be several orders of magnitude lower than Cd(II) or Zn(II) affinity [14]. To rule out low-affinity divalent metal ion binding by His-40 and His-41 in ZF2, we recorded spectra of Cd(II)–peptide complexes (not

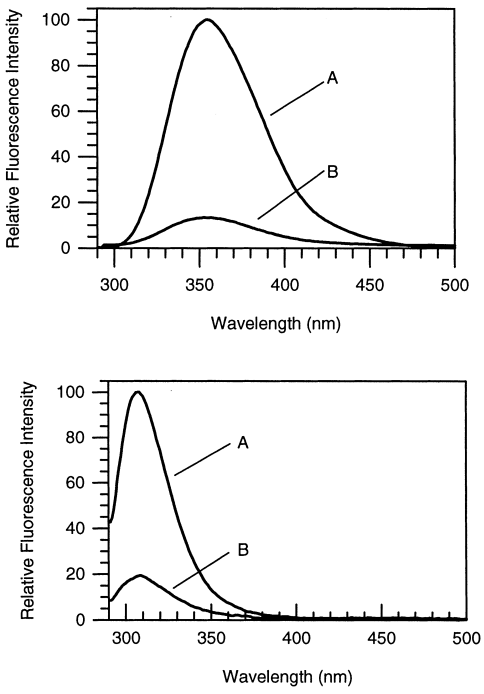

Fig. 3. Fluorescence emission spectrum of double ZF (10 μM in 10 mM sodium phosphate, pH 7, top panel) and $ZF2^Y$ (10 μM in 10 mM sodium phosphate, pH 7, bottom panel) before (A) and after (B) Cys alkylation with NEM. Excitation was at 280 nm for the double ZF and at 274 nm for $ZF2^Y$. Excitation bandwidth was 1.5 nm and emission bandwidth was 5 nm in each case. Cuvettes were 2×10 mm quartz; excitation path length was 2 mm.

shown). A charge transfer band was observed for both DZF and $ZF2^Y$, but not $ZF2^S$, supporting our contention that the extra His residues in ZF2 do not bind metal ions. Chelation of Cd(II) or Zn(II) ions, however, has been observed with synthetic peptides spanning the first or second ZFs of HIV-1 NC protein upon replacement of all their constituent Cys residues by His (P. Nower and J. Casas-Finet, unpublished results). These observations strongly suggest that the lack of metal ion chelation in $ZF2^S$ is not inherent in the composition of the metal cluster, but in the improper spacing of the chelating residues.

3.4. *Effect of Cys alkylation on double ZF and $ZF2^Y$ fluorescence*

To test whether the adjacent arrangement of the ZF motifs in visna p8 would affect their metal ion coordination, we studied a double ZF peptide (p8 purified from viral suspensions was available in insufficient amounts for these experiments). Absorbance spectra of the double ZF in the presence of Co(II) showed near-UV and visible bands with peak wavelength identical to those shown in Fig. 1 for ZF1 and ZF2 (not shown). This result suggests that no reorganization of the metal clusters occurs due to apposition of the two ZF motifs.

Although Zn(II) ions are the natural ligand of NC proteins in vivo, they are not easily studied by absorbance methods. The intrinsic fluorescence of Zn(II)-bound double ZF and $ZF2^Y$ peptides, however, decreased greatly after NEM reaction (Fig. 3). This effect is due to deactivation of the aromatic side chain, Tyr in $ZF2^Y$, Trp in the double ZF. Deactivation may result from collision with nearby residues, after the ZF domain unfolds in the absence of bound metal ion. Unfolding of these Zn(II)-bound peptides upon NEM reaction was also demonstrated by circular dichroism spectra (data not shown). These results show that the replacement of Zn(II) by Cd(II) or Co(II) does not alter the binding arrangement about the chelated metal ion.

3.5. *Effect of DNA on double ZF fluorescence*

Since we have established that the two extra His in ZF2 do not participate in the chelation of metal ions, we proceeded to test for other functions. In other retroviral NC proteins, aromatic residues in this region are known to interact with nucleic acids, forming stacked structures with nucleic acid bases [6–8]. It is conceivable that the planar, hydrophobic, imidazole ring could fulfill a similar role. We took advantage of the fluorescence emission of the double ZF (3 μM) to monitor the effect induced upon addition of the deoxyoligonucleotide dI_8 (to 80 μM, a 26-fold excess) (Fig. 4). The significant quenching observed (53%) is consistent with intercalation of Trp-21, present in the first ZF motif of the double ZF peptide, with nucleic acid bases. NaCl addition reversed the effect, showing that DNA binding had an electrostatic component. Similar results were seen with the tyrosine emission of $ZF2^Y$. Hence, both ZF motifs are capable of binding DNA.

3.6. *p8 fluorometric titrations*

Viral p8 was titrated with single-stranded DNA, both poly- and oligonucleotides, in 10 mM phosphate buffer, pH 7. Fluorescence emission from p8 is due to Tyr-21 in the first ZF region. Fluorescence quenching (30–60%) in the presence of saturating amounts of nucleic acid (Fig. 5) shows involvement of the first ZF of p8 in DNA binding. The occluded binding site size was 7±2 bases for single-stranded DNA; consequently, p8 bound octanucleotides with 1:1 stoichiometry (Fig. 5). Single retroviral ZF peptides occlude up to three bases [12]; thus, both ZFs in visna p8 are likely to be involved in DNA binding.

Salt back-titrations indicated a net displacement of 3–4 counterions from the DNA upon p8 binding. Preliminary results yield a DNA binding affinity in the micromolar range for the interaction of visna p8 with homopolymeric octanucleotides of various base composition. Extrapolation to standard thermodynamic conditions (1 M NaCl) showed a significant

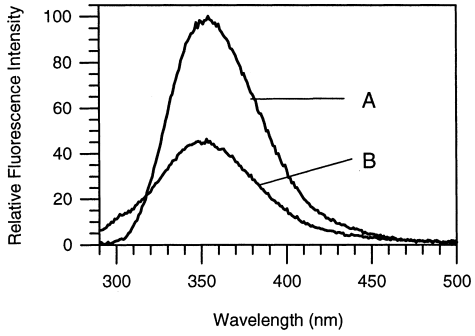

Fig. 4. Effect of DNA on fluorescence emission of the p8 double ZF (3.2 μM, A) alone and in the presence of 80 μM dI_8 (B). Excitation wavelength was at 280 nm with 1.5 nm bandwidth; emission bandwidth was 5 nm. Cuvettes were 2×10 mm quartz; excitation path length was 2 mm. Nucleotide screening was not significant, since ε_{280} of inosine is $\sim$2000 cm^{-1} M^{-1}. This yields a screening factor no greater than 4%.

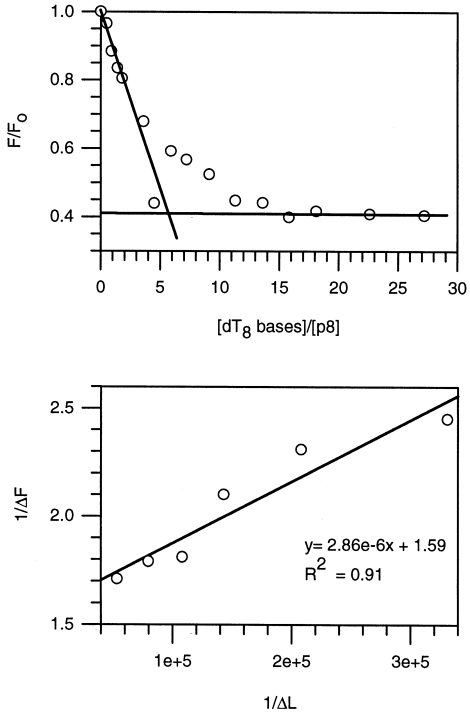

Fig. 5. Equilibrium binding isotherm of visna p8 (1.7 μM) with dT_8 (top panel), and Scatchard analysis (bottom panel). Occluded binding site size, limiting fluorescence quenching and binding affinity can be estimated from the titration plots.

hydrophobic contribution to the binding free energy. The contribution of non-electrostatic interactions to the overall ΔG under physiological ionic strength conditions ranged between ~30% for T and U and ~45% for C. The relative contribution of non-electrostatic interactions to the overall binding free energy is similar to that seen for the NC proteins of HIV-1 and SIV [6,12] in which aromatic side chains in both ZF domains participate simultaneously in the binding process. This leads us to postulate that the two additional histidine residues (H40, H41) in the second ZF of p8 participate in nucleic acid binding, given the paucity of other hydrophobic side chains in this motif.

4. Conclusions

ZF domains (**Cys**-X_2-**Cys**-X_4-**His**-X_4-**Cys**) in retroviral NC proteins usually contain one histidine residue, which forms part of the metal ion coordination complex. Visna p8 has two additional histidines between the first two Cys residues of its ZF2. The role of these His residues was assessed by UV-Vis and fluorescence spectroscopy.

Absorption spectra of Co(II)–ZF complexes revealed charge transfer absorption bands at ~309 nm and *d-d* transitions at 550–750 nm. The λ_{max} and line shape of the *d-d* splittings were identical for ZF1, ZF2 and $ZF2^Y$ (a mutant in which the two extra histidines were replaced by tyrosine). These results point to an identical metal cluster composition in all three peptides. Neither charge transfer nor *d-d* transition peaks were seen in $ZF2^S$, a peptide with cysteine residues replaced by serine, in the presence of Co(II).

Spectra were also taken after reacting peptides with the Cys-alkylating agent NEM. NEM abolished the optical features mentioned above and quenched the fluorescence of $ZF2^Y$ and Trp-labeled double ZF by ~85%. This shows that cysteine residues are crucial for divalent metal ion binding.

Zn(II)-bound double ZF and $ZF2^Y$ fluorescence was quenched in the presence of DNA. The occluded site size suggests that both ZFs participate in the association of p8 with nucleic acids. The extent of hydrophobic contribution to ΔG suggests that stacking of amino acid side chains with nucleic acid bases takes place in each of the ZF motifs.

In summary, our results demonstrate that, in visna NC p8, the ZF2 metal ion coordination sphere is composed of three cysteines and one histidine in the canonical spacing, and suggest that His-40 and His-41 interact with nucleic acid bases in p8–DNA complexes. In agreement with this hypothesis, we have found that among the gene sequences available from the PIR database, His-40 is absolutely conserved and His-41 exhibits 60% conservation (PIR1: FOLJVS, A45390, A46335, PIR2: JQ1161). In double-ZF proteins, an aromatic residue is usually found immediately after the first cysteine in the metal cluster. In the case of HIV-1 NC [12] and SIV NC [6] the aromatic residue has been shown to be functional in nucleic acid binding. It is tempting to speculate that His-40 assumes an identical role.

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References

[1] Rein, A., Henderson, L.E. and Levin, J.G. (1998) Trends Biochem. Sci. 23, 297–301.
[2] Darlix, J.-L., Lapadat-Tapolsky, M., de Rocquigny, H. and Roques, B.P. (1995) J. Mol. Biol. 254, 523–537.
[3] Henderson, L.E., Copeland, T.D., Sowder, R.C., Smythers, G.W. and Oroszlan, S. (1981) J. Biol. Chem. 256, 8400–8406.
[4] Berg, J.M. (1986) Science 232, 485–487.
[5] Bess Jr., J.W., Powell, P.J., Issaq, H.J., Schumack, L., Grimes, M.K., Henderson, L.E. and Arthur, L.O. (1992) J. Virol. 66, 840–847.
[6] Urbaneja, M.A., McGrath, C.F., Kane, B.P., Henderson, L.E. and Casas-Finet, J.R. (2000) J. Biol. Chem. 275, 10394–10404.
[7] Wu, J.Q., Ozarowski, A., Maki, A.H., Urbaneja, M.A., Henderson, L.E. and Casas-Finet, J.R. (1997) Biochemistry 36, 12506–12518.
[8] Wu, J.Q., Maki, A.H., Ozarowski, A., Urbaneja, M.A., Henderson, L.E. and Casas-Finet, J.R. (1997) Biochemistry 36, 6115–6123.
[9] Braun, M.J., Clements, J.E. and Gonda, M.A. (1987) J. Virol. 61, 4046–4054.
[10] Sonigo, P. et al. (1985) Cell 42, 369–382.
[11] Henderson, L.E., Sowder, R., Smythers, G., Benveniste, R.E. and Oroszlan, S. (1985) J. Virol. 55, 778–787.
[12] Urbaneja, M.A., Kane, B.P., Johnson, D.G., Gorelick, R.J., Henderson, L.E. and Casas-Finet, J.R. (1999) J. Mol. Biol. 287, 59–75.
[13] Lohman, T.M. and Mascotti, D.P. (1992) Methods Enzymol. 212, 424–458.
[14] Krizek, B.A., Merkle, D.L. and Berg, J.M. (1993) Inorg. Chem. 32, 937–940.
[15] Maret, W. and Vallee, B.L. (1993) Methods Enzymol. 226, 52–71.
[16] Shi, Y., Beger, R.D. and Berg, J.M. (1993) Biophys. J. 64, 749–753.