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Electrostatic interactions in hirudin–thrombin binding

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Abstract

Hirudin is a good anticoagulant owing to potent inhibition of the serine protease thrombin. An aspartate- and glutamate-rich portion of hirudin plays an important part in its tight binding to thrombin through a ladder of salt bridges, and these residues have previously been mutated to asparagine or glutamine. Detailed calculations of the electrostatic contribution to changes in binding from these mutations have been performed using the finite-difference Poisson–Boltzmann method which include charge–charge interactions, solvation interactions, the residual electrostatic interaction of mutant residues, pK_a shifts, and ionic strength. Single mutant effects on binding energy were close to experimental values, except for the D55N mutant whose effect is overestimated, perhaps because of displacement of a bound chloride ion from the site where it binds. Multiple mutation values were generally overestimated. The effect of pK_a shifts upon the binding is significant for one hirudin residue E58, but this appears to be due to a poor salt bridge with thrombin caused by crystal contacts. Electrostatic interaction between the acidic residues is unfavorable. However, analysis of experimental multiple mutation/single mutation data shows apparently negative interactions between these residues, from which it is concluded that structural changes can occur in the complex to relieve an unfavorable interaction when more than one acidic residue is mutated. In all cases, there is a loss in stability of the complex from mutations due to loss of favorable charge–charge interactions with thrombin, but this is largely compensated for by reduced unfavorable desolvation interactions, and by residual polar interactions in the Asn/Gln mutants.

Keywords: Hirudin; Thrombin; Electrostatics; Binding; Finite Difference; Poisson–Boltzmann

1. Introduction

Hirudin is a 65-amino-acid protein which is a potent inhibitor of the plasma serine protease thrombin and hence it is a powerful anticoagulant [1,2]. Binding of hirudin and thrombin has been extensively studied [3–5] and the structure of the hirudin–thrombin complex has been determined by X-ray crystallography [6]. Hirudin contains an aspartate- and glutamate-rich region, residues 55–62, which is important for tight binding through its interaction with a basic region on thrombin outside the active site, the fibrinogen recognition site [4,7]. Thrombin also has a sodium-binding site opposite the active site [8] which is involved in the slow/fast binding transition exhibited by thrombin [9]. The crystal structure of the hirudin–thrombin complex reveals that the acidic carboxy terminus region of hirudin lies in an extended conformation along a cleft in thrombin, and that there are interactions between residues D55, E57, and E58 of hirudin and

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the parallel residues R73, R75, R77A and K149E of thrombin to form a 'ladder' of salt bridges [6]. There are also other electrostatic interactions in this region. Interestingly, the neighboring residues E57 of hirudin and R75 of thrombin lie close to a crystallographic two-fold axis of symmetry, and instead of making a salt bridge with each other, as their flanking pairs E55–R73 and E58–R77A do, they each make a salt bridge with the corresponding E57 and R75 residues on the neighboring complex in the crystal that is related by the crystallographic two-fold axis, forming a swapped-partner salt bridge [6].

The five hirudin residues D55, E57, E58, E61, and E62 have all been mutated, singly and in combination to glutamine (asparagine for D55), and the binding energy measured for the wild-type and mutant hirudin binding to thrombin [5,10]. The effect of such mutations is likely to be largely electrostatic since a formally charged residue is being replaced by a neutral residue of the same size which can still make hydrogen bonds. The finite-difference Poisson–Boltzmann (FDPB) method can account for shape, charge distribution, dielectric and ionic-strength effects in proteins, and so is a potentialy suitable method for incorporating structural detail at an atomic level into binding-energy calculations. Previous successful applications of the FDPB method to study binding energies in a variety of complexes include calculating the electrostatic contribution to binding in protein–protein, protein–ligand, drug–DNA and protein–DNA interactions [11–17]. The hirudin–thrombin system would also seem to be a suitable system to apply the FDPB method. In fact, the electrostatic contribution to the binding energy for such mutations has been calculated previously [10] using the FDPB method, and also using the modified Tanford–Kirkwood (MTK) method [18] which models the structure in less detail, but requires less computation. It was found that the FDPB method greatly overestimated the effect of the mutations, while the MTK method provided more reasonable results [10]. However, examination of these calculations shows that only the charge–charge interaction between the mutated residue and the rest of the charges on hirudin and thrombin was included in the FDPB calculations. The contribution of the solvation, or self-energy of the mutated residue was not included, although this can be as large as the charge–charge interaction [19]. The goal of the current study is to apply the FDPB method in detail to the calculation of the hirudin–thrombin binding, specifically the effect of the mutations D55N, E57Q, E58Q, E61Q and E62Q, and include the solvation contribution from the mutated residues. In addition, there has been recent progress in the application of the FDPB method to protein electrostatics, including the development of accurate partial-charge sets for use with the FDPB method, particularly the PARSE (parameters for solvent energy) set [20]. The use of partial charges also allows for the inclusion of residual electrostatic interaction in the mutant proteins, since asparagine and glutamine, though neutral, have considerable polar electrostatic character. The role of crystal contacts in the region of the mutations is also examined to see whether changes in local structure compared to in solution might affect the calculated binding free-energy changes

2. Methods

2.1. Protein structure

The structure of the hirudin–thrombin complex has been determined to 2.3 Å resolution by Rydel et al. [6]. The coordinates of this structure were obtained from entry 4HTC of the Brookhaven protein database [21]. The structure is missing the N-terminal 2 residues and the C-terminal residue of thrombin, and the first residue after the thrombin cleavage site because of disorder in the crystal. The loop 33–35 of hirudin is also disordered.[6]. All these regions are far from the acidic C-terminal domain of hirudin, and their omission is unlikely to affect the binding-energy calculations. Hydrogens were added using *Insight* (Biosym Corporation, San Diego), and the structure minimized for 500 steps using steepest-descent minimization in *Discover* (Biosym Corporation, San Diego) to anneal any steric strain and refine the positions of the added hydrogens.

In the crystal structure there are a series of salt bridges between the acidic residues in the hirudin C-terminal region and arginine and lysine residues of thrombin. Asp 55 of hirudin forms a good salt bridge with Arg 73 of thrombin. Glu 58 forms a salt bridge with Arg 77A of thrombin. However, the intervening

Glu 57 of hirudin and Arg 75 of thrombin, although in close enough, do not form a good salt bridge, the carboxyl-to-guanidine distance being approximately 6 Å. Instead, Arg 75 makes a salt bridge to Glu 57 of hirudin from the neighboring hirudin–thrombin complex in the crystal lattice. Similarly, the Glu 57 makes a salt bridge with Arg 75 of the neighboring complex. The four residues involved in this salt-bridge partner-swapping are arranged around a crystallographic two-fold axis. The Glu 58–Arg 77A salt bridge also has less than optimal geometry because of a lattice contact, in this case thrombin Ser 153 of a lattice neighbor which makes a hydrogen bond with Glu 58 (Fig. 1). The possibility arises that optimal salt-bridge geometries within the complex in solution are sacrificed to promote better lattice contacts in the crystal. A second wild-type-complex structure was thus generated by optimizing the Arg 77–Asp 57 and Arg 77A–Glu 58 salt-bridge geometries. This was done using the builder module with the bump-check option within *Insight* to rebuild conformations for these four sidechains that are both sterically reasonable and have close to ideal salt-bridging (i.e. H-bonding with a 2.8–3.0 Å heavy-atom distance, and close to linear O–H–N angle [22]). Mutant-hirudin structures were generated by simply replacing either the Asp or Glu by Asn or Gln respectively. Since the relacements are essentially isosteric, no steric strain is introduced by these mutations. Ambiguity often exists about the relative orientation of the amide group in the sidechain of Asn and Gln residues even in known structures. Therefore, mutant structures were built with alternative orientations for each mutation (positions of the OD and ND atoms interchanged for Asn, or OE and NE atoms interchanged for Gln). The conformations where the amide nitrogen takes the position of the OD2/OE2 atom are designated conformation A. Conformation B refers to the case where the nitrogen occupies the OD1/OE1 position. These are arbitrary designations since the labelling of the two equivalent oxygens of the carboxyl group in the original PDB

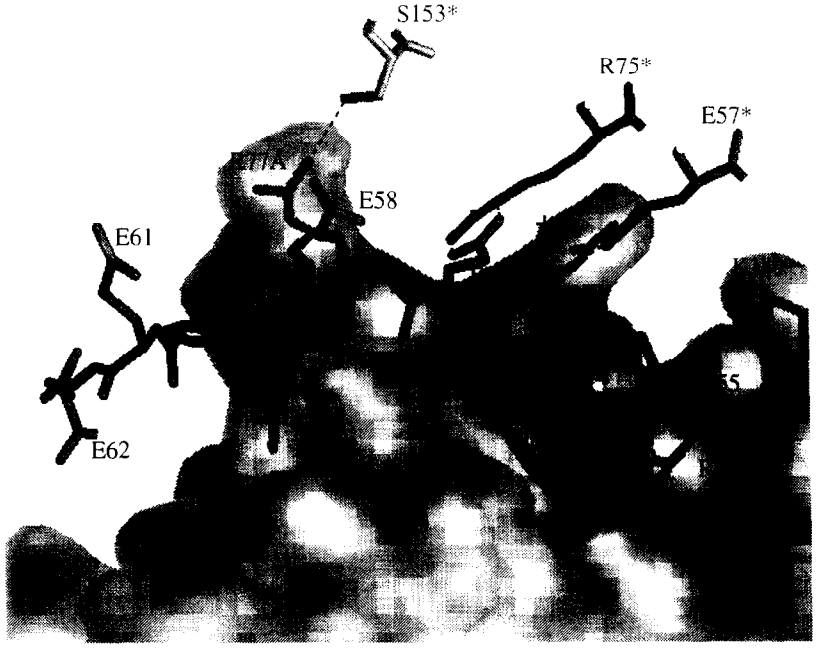

Fig. 1. Structure of the thrombin fibrinogen binding site-hirudin acidic region binding in the crystal structure. The molecular surface of thrombin in the second binding site region is shown, along with residues 73, 75, 77A and 149 in bond representation. Acidic region of hirudin, residues 55–62, is shown in bond representation. Residues from the neighboring complex in the crystal lattice are denoted by asterisked residue numbers. Arg75 forms a salt bridge with Glu57* and vice versa. Key salt bridge hydrogen bond interactions are indicated by dashed lines. Picture is produced by GRASP [24].

file is arbitrary. Binding-energy calculations were performed for both A and B conformations for all mutations. The orientation of the mutated residue that produced the lowest energy in the bound state was taken to be the relevent one for binding-energy calculations as presumably this is the most populated, or likely, conformation.

2.2. Binding-energy calculations

The binding-energy changes produced by the mutations D55N, D57N, E58Q, E61Q, E62Q were assumed to be primarily electrostatic. The residue replacements are essentially isosteric, so that van der Waals and hydrophobic energy changes are likely to be negligible. No contribution from possible structural changes was included, i.e. the same structures of hirudin and thrombin were used in the bound and free states. While it is possible that structural changes occur, prediction of these changes would involve extensive exploration of protein conformational space. This lies outside of the scope of the present study, which is aimed at seeing how much of the effect of the mutations can be accounted for by purely electrostatic effects.

Electrostatic energies were calculated using the FDPB method implemented in the software package DelPhi [19,23,24]. Parameters used in the FDPB calculations were as follows: Grid dimensions were $65 \times 65 \times 65$, with a final scale of about 1 grid per Å. Solutions were obtained for the linear Poisson–Boltzmann equation with Debye–Hückel-type boundary conditions [23] using the multigridding method of iteration [25,26] with a final convergence value of $1 \times 10^{-4} kT/e$ total residual error in the potential. Binding-energy contributions are obtained by performing FDPB calculations on the bound- and free-state proteins and taking the difference in energies. For each binding-energy calculation, 27 FDPB runs were performed with the protein(s) mapped onto the lattice in a different position, and the average and standard deviation of the electrostatic energies computed. This translational averaging reduces the error due to the finite resolution of the lattice, and provides an estimate of the numerical precision of the calculations. For clarity, estimates of the numerical precision are tabulated only for selected results, and are representative for these kind of calculations. The solvent and protein dielectric constants used were 80 and 4 respectively. The salt concentration was 0.15 M [5]. Radii and charge values were taken from the PARSE parameter set [20]. The PARSE charge values for the wild type and mutated residues 55, 57, 58, 61 and 62 are given in Table 1. For comparison with previous calculations by Karshikov et al.[10], a formal charge set was also used (charges placed on on Arg, Lys, Asp, Glu His sidechains and peptide terminii only, see Table 1). All ionizable residues were assumed to be fully ionized, corresponding to a pH of around seven with close to normal pK_a values for the charged residues. In addition, pK_a shifts of the residues D55, E57, E58, E61, E62 due to electrostatic effects were obtained from FDPB calculations [27], see below.

Table 1
Atomic charges used for electrostatic calculations [a]

Set	Atoms (residue)	Charge
Formal	OE1, OE1 (Glu^-) OD1, OD2 (Asp^-)	−0.5
	NZ (Lys)	+1.0
	NH1, NH2 (Arg)	+0.5
	N (N-terminus)	+1.0
	OXT1, OXT2 (C-terminus)	−0.5
PARSE [b]	OE1, OE1 (Glu^-) OD1, OD2 (Asp^-)	−0.55
	CD (Glu^-), CG (Asp^-)	+0.10
	CD (Gln), CG (Asn)	+0.55
	OE1 (Gln), OD1 (Asn)	−0.55
	NE2 (Gln), ND2 (Asn)	−0.78
	HE21, HE22 (Gln), HD21, HD22 (Asn)	+0.39
	OE1, OE1 ($Glu^- H^+$) OD1, OD2 ($Asp^- H^+$)	−0.495
	HE2 (Glu), HD2 ($Asp^- H^+$)	+0.44
	CD (Glu), CG ($Asp^- H^+$)	+0.55

[a] Charge given in proton charge units.
[b] Taken from the PARSE charge set. Values for the mutated residues sidechains only are reproduced here. Charges used for all other sidechains and the backbone are given in Sitkoff et al. [20].

The total electrostatic free energy of a molecule or complex is obtained from the FDPB calculations using the equation

$$G_{\text{tot}}^{\text{elec}} = \frac{1}{2} \sum_i q_i \phi_i \qquad (1)$$

where the sum is over all the atomic charges, q_i, in hirudin and thrombin and ϕ_i is the total potential at that charge due to all other charges, solvent screen-

ing, and the reaction potential induced by that charge [19]. The difference in electrostatic free energy upon binding is thus given by $\Delta G_{\mathrm{tot}}^{\mathrm{elec}} = \frac{1}{2}\sum_i q_i \Delta\phi_i$, where $\Delta\phi_i$ is the change in potential upon binding. In the case where we are interested in the contributions of say two particular residues i and j to the binding energy, this may be written

$$\Delta G_{\mathrm{wt}}^{\mathrm{elec}} = \frac{1}{2}\sum_i q_i \Delta\phi_{ii} + \frac{1}{2}\sum_j q_j \Delta\phi_{jj} + \frac{1}{2}\sum_p q_p \Delta\phi_{pp} + \sum_j q_j \Delta\phi_{ij} + \sum_p q_p \Delta\phi_{ip} + \sum_p q_p \Delta\phi_{jp} \tag{2}$$

where $\Delta\phi_{xy}$ refers to a difference in potential between the complex and dissociated state, arising from the set of charges x, at a set of charges y. Subscripts i, and j refer to the atomic charges/locations on the residues i and j, in this case any of the mutated residues 55, 57, 58, 61, or 62 on hirudin. The subscript p refers to the rest of the charges/locations on hirudin and thrombin. The first three terms of Eq. (2) describe the solvation, or reaction field energy contributions to the electrostatic binding free energy. These terms require evaluation of the potential at atomic positions to which charge is assigned. In the FDPB method, this potential contains a finite, but meaningless grid energy contribution which is removed by performing the electrostatic calculations on the complex and dissociated proteins using the same lattice mapping, and taking differences in potentials [13]. The last three terms describe the charge–charge interaction, including the solvent screeing effect, between residues i and j, residue i and the rest of the charges, and residue j and the rest of the charges respectively. If residue i is mutated, the electrostatic free energy is now

$$\Delta G_{\mathrm{wt}}^{\mathrm{elec}} = \frac{1}{2}\sum_i q_i^* \Delta\phi_{ii}^* + \frac{1}{2}\sum_j q_j \Delta\phi_{jj} + \frac{1}{2}\sum_p q_p \Delta\phi_{pp} + \sum_j q_j \Delta\phi_{ij}^* + \sum_p q_p \Delta\phi_{ip}^* + \sum_p q_p \Delta\phi_{jp} \tag{3}$$

where the asterisked charges denote those of the mutated residue i, and the asterisked potentials those arising from the mutated residue. The change in binding energy due to the mutation is

$$\Delta\Delta G_{\Delta i}^{\mathrm{elec}} = \frac{1}{2}\sum_i \left(q_i^* \Delta\phi_{ii}^* - q_i \Delta\phi_{ii} \right) + \sum_j q_j \left(\Delta\phi_{ij}^* - \Delta\phi_{ij} \right) + \sum_p q_p \left(\Delta\phi_{ip}^* - \Delta\phi_{ip} \right) \tag{4}$$

Similarly, for mutation of residue j, we have

$$\Delta\Delta G_{\Delta i}^{\mathrm{elec}} = \frac{1}{2}\sum_j \left(q_j^+ \Delta\phi_{jj}^+ - q_j \Delta\phi_{jj} \right) + \sum_i q_i \left(\Delta\phi_{ji}^+ - \Delta\phi_{ji} \right) + \sum_p q_p \left(\Delta\phi_{jp}^+ - \Delta\phi_{jp} \right) \tag{5}$$

where the superscript $^+$ denotes mutant j residue quantities. For a double mutation of i and j

$$\Delta\Delta G_{\Delta i,\Delta j}^{\mathrm{elec}} = \frac{1}{2}\sum_i \left(q_i^* \Delta\phi_{ii}^* - q_i \Delta\phi_{ii} \right) + \frac{1}{2}\sum_j \left(q_j^+ \Delta\phi_{jj}^+ - q_j \Delta\phi_{jj} \right) + \sum_i \times \left(q_i^* \Delta\phi_{ji}^+ - q_i \Delta\phi_{ji} \right) + \sum_p \left[\left(\Delta\phi_{ip}^* + \Delta\phi_{jp}^+ \right) - \left(\Delta\phi_{ip} + \Delta\phi_{jp} \right) \right] \tag{6}$$

It should be noted that from reciprocity $q_i \Delta\phi_{ji} = q_j \Delta\phi_{ij}$ for the wild type, and similar relations hold for each of the mutants. Using this, Eq. (6) may be written in terms of the single-site mutation values

$$\Delta\Delta G_{\Delta i,\Delta j}^{\mathrm{elec}} = \Delta\Delta G_{\Delta i}^{\mathrm{elec}} + \Delta\Delta G_{\Delta j}^{\mathrm{elec}} + \sum_j \left(q_i \Delta\phi_{ji} + q_i^* \Delta\phi_{ji}^+ - q_i \Delta\phi_{ji}^* - q_j \Delta\phi_{ij}^* \right) \tag{7}$$

The third term of Eq. (7) accounts for the non-additivity of the single site mutation values, and it arises from the difference in interaction between the two mutated residues in the bound and dissociated states. If this interaction between the two residues is small except in the wild type where they are both charged, the third term simplifies to $\sum_i q_i \Delta\phi_{ji}$. In this simplified case, the origin of the non-additivity term is evident: it arises from the fact that in each single site mutation the i–j interaction is lost, so in simply summing the two energies to get the double mutant value, this loss would be counted twice. Adding this term back provides the correct double mutant value. The full Eq. (7) accounts for this residue–residue interaction when interaction between residues in the mutants cannot be neglected. For a more general discussion of this kind of analysis and double mutant cycles, particularly in the context of experimental design and analysis, see for example Horovitz and Fersht [28]. Multiple mutant cases can be treated with Eqs. (6 and 7) by considering all the pairwise mutation interactions and summing the effects.

It should be noted that in a previous analysis of the hirudin–thrombin binding [10] while the inital expression for the total electrostatic energy is identical to Eq. (1) (Eq. (2) of Ref. [10]), the final expression they used to calculate the effect of single site mutations (Eq. (5) Ref. [10]) does *not* include the self energy or solvation term of the mutated residue (The first term of Eq. (4) above). Similarly their multiple mutation expression (Eq. (4) of Ref. [10], Eq. (7) here) has no solvation term, as indicated by the lack of a term with a factor of one half, and exclusion of terms of the form $q_i\phi_i$ from their summation. Thus, these previous calculations, unlike those presented here, contain only the interaction of the mutated residue with the charges on thrombin and other charges on hirudin, and the effect of solvent on these two interactions. In addition to these differences, the current study extends the previous study by taking into account the polar nature of the mutated residues, assigning charge to Asn and Gln sidechains, whereas previously, it was assumed that there was no charge on the mutated residues. This is made possible by the availability of the PARSE parameter set, which has been specifically parameterized for use with the FDPB method, and which has explicit representation of the charges for polar residues like Asn and Gln.

2.3. pK_a Shifts

If the pK_a of any of the residues 55, 57, 58, 61, 62 in hirudin is shifted in either the bound or free states to a value close to the pH, then uptake or release of protons can occur. This would potentially affect the calculated value of the mutation. For example, mutation from the protonated form or Asp to Asn is less drastic than from ionized form. Thus, both the protonated and unprotonated species would have to be considered in the binding-equilibrium calculations. Since pK_a shifts occur primarily from electrostatic effects, they can be calculated using the FDPB method along with the electrostatic binding free energy contributions [27,29]. The pK_a shifts for the five mutated acidic residues were obtained as

$$pK_a = pK_a^o + \left(\frac{1}{2.303kT}\right) \times \left(\frac{1}{2}\sum_i \left(q_i\Delta\phi_{ii} - q_i^u\Delta\phi_{ii}^u\right) + \sum_p q_p\left(\phi_{ip} + \phi_{ip}^u\right)\right) \quad (8)$$

where pK_a^o is the reference state pK_a for that residue (3.85 for Asp and 4.25 for Glu). The superscript u refers to the unionized, or protonated state of the residue, unsuperscripted quantities refer to the ionized state as in Eqs. (1)–(7). For the pK_a calculations, however, $\Delta\phi_{ii}$ is the difference in the residue's reaction potential between the reference state (isolated in water [27,29]), and in the protein. As before, ϕ_{ip} is the potential from the residue at other protein charges, summed over all other residues. Eq. (8) is applied to both the bound and unbound hirudin to calculate pK_a shifts of the mutated residues upon binding. For the protonated form of the sidechain in Eq. (8), there is a choice of whether to put the proton on the OD1/OE1 or OD1/OD2 positions. As with the positioning of the Gln and Asn nitrogen, FDPB calculations were performed for both cases, and the position which resulted in the lowest electrostatic energy in the complex was used. In the pK_a calcula-

Table 2
Interaction between mutated residues on hirudin [a]

Residue		E57	E58	E61	E62
D55	Complex	0.3	0.1	0.0	0.2
	Hirudin	0.1	0.1	0.0	0.1
	Difference	0.1	0.1	0.0	0.1
E57	Complex		0.6	0.1	0.0
	Hirudin		0.3	0.0	0.0
	Difference		0.3	0.0	0.0
E58	Complex			0.2	0.1
	Hirudin			0.1	0.1
	Difference			0.1	0.0
E61	Complex				0.2
	Hirudin				0.2
	Difference				0.0

[a] Values in kcal mol^{-1}. All interactions were calculated for the fully ionized residues in wild-type hirudin, using PARSE charges. The change in interaction between any two of these residues upon binding when either or both were mutated to Asn/Gln was negligible.

tions, other ionizable residues are again assumed to have unperturbed pK_a's. A full self-consistent calculation of all pK_a's (see Yang et al. [27], Bashford et al. [30], and Gilson [31]) is a feasible but nontrivial calculation, and was not attempted here.

In the case of a significantly upward shifted pK_a in the bound or free hirudin, the protonation equilibrium contribution of the ith residue's part of the electrostatic binding energy is given by

$$\Delta\Delta G_i^{\text{prot}} = \Delta\Delta G_i + kT\ln \times \left(\frac{1+\exp\left[2.303kT\left(pK_a^b - pH\right)\right]}{1+\exp\left[2.303kT\left(pK_a^d - pH\right)\right]} \right) \quad (9)$$

where $\Delta\Delta G_i$ if that residue's contribution in the completely ionized state, and pK_a^b and pK_a^d are its pK_a's in the bound and dissociated states.

3. Results

The electrostatic interaction between the five mutated hirudin residues 55, 57, 58, 61 and 62 was calculated for the wild-type protein and the five single site mutants. Interaction energies for the wild-type protein are given in Table 2 for the bound and dissociated hirudin, and for the change upon binding. Most of the interactions are small, with the largest being 0.3 kcal mol^{-1} for the E57–E58 interaction. Interactions between any mutated residue and the other four wild-type or mutated residues was negligible (results not shown), so that the non-additivity contribution given by Eq. (7) simplifies to $\sum_i q_i \Delta\phi_{ji}$, the change in charge–charge interaction between the two carboxylate groups upon binding, where q_i are the charges of residue i, and $\Delta\phi_{ji}$ is the change in potential upon binding at these charges due to residue j.

Table 3 shows results from the pK_a calculations. The pK_a shifts for E57 and E58 were calculated using both the crystal structure and the optimized salt-bridge structure. For the former, little difference was found between the two results. In all cases except E58, the pK_a is shifted down in both free hirudin and the complex. In these cases at physiological pH, there is thus no contribution from the protonated form to the binding energy. For E58, however, in the original structure the pK_a is shifted

Table 3
pK_a shifts induced in hirudin carboxylate groups

		Residue						
		D55N	E57Q [a]	E57Q [b]	E58Q [a]	E58Q [b]	E61Q	E62Q
Free	ΔpK_a solvation	0.96	0.42	0.58	0.67	0.11	0.13	0.58
	ΔpK_a charge–charge	−1.15	0.63	0.50	0.70	0.52	0.21	−0.03
	pK_a	3.66	5.31	5.32	5.63	4.88	4.59	4.80
Bound	ΔpK_a solvation	3.76	2.35	3.74	3.74	1.36	0.13	0.62
	ΔpK_a charge–charge	−6.74	−3.75	−6.06	0.11	0.06	0.05	−0.35
	pK_a	0.87	2.85	1.93	8.10	5.67	4.43	4.52

[a] Using crystal structure.
[b] Using optimized E57 and E58 salt bridges.

Table 4
Interaction between mutated residue and hirudin/thrombin charges [a]

	Residue				
	D55	E57	E58	E61	E62
Complex	−9.0	−0.6	−2.4	0.2	−0.4
Hirudin	0.3	0.8	0.9	0.5	0.5
Difference [b]	9.2 ± 1	1.5 ± .03	3.3 ± 0.3	0.3 ± 0.1	0.8 ± 0.2
Ref. [10] [c]	9.1	1.7	2.6	0.3	1.0
Experiment [c]	0.63	1.41	1.26	0.31	0.67

[a] Values in kcal mol^{-1} for the electrostatic interaction free energy of given residue with the other charged residues in the complex or with hirudin alone. Calculated using the crystal structure and a formal charge set for both mutated residues and other hirudin and thrombin residues. Ionic strength is 0.125M.
[b] Given as (complex−hirudin), i.e. as the expected change in binding energy from a single site mutant if no other interaction such as desolvation were present. Numerical error is estimated from the standard deviation of 27 FDPB runs with different molecular positions on the lattice.
[c] Previous FDPB estimates and experimental values taken directly from Table 2 of Karshikov et al. [10].

to 8.1 in the bound form, due primarily to unfavourable desolvation, which is insufficiently compensated by any charge–charge interaction, especially in the absence of a true salt bridge with thrombin Arg77A. The pK_a is shifted high enough that the protonation/deprotonation equilibrium would contribute significantly to the binding energetics. With the optimized salt bridge, the pK_a is still shifted but by a smaller amount, resulting in a pK_a sufficiently below the pH that there is little protonation contribution.

The charge–charge contribution to changes in binding energy upon single site mutations is given in Table 4. For comparison with previous calculations (Ref. [10], data reproduced in the table), the formal charge set was used. The current calculations give essentially the same results as previously, small differences presumably arising from the slight differences in structure resulting from the minimization/proton placement. In all cases except E62, the charge–charge interaction alone is larger than the experimentally observed binding-energy change, the point previously made by Karshikov et al. [10]. In Table 5, more detailed calculations of the single site mutant binding free energy changes are presented. These now include the solvation contribution of the mutated residue, the interaction with the

Table 5
Electrostatic free energy contributions to binding—effect of single-site mutations [a]

Protein	Contribution	Residue				
		D55	E57	E58	E61	E62
Wild type	Solvation [b]	4.5	5.3	2.1	0.0	0.1
	Charge–charge [c]	−10.8	−7.1	−2.6	−0.2	−0.9
Mutant	Solvation	0.8	1.1	0.3	0.0	0.0
(orientation A [d])	Charge—charge	−3.5	−1.9	1.0	0.0	0.0
Mutant	Solvation	0.9	0.6	0.6	0.0	0.0
(orientation B [d])	Charge − charge	0.7	1.3	0.5	0.0	−0.1
Difference	Solvation [e]	−3.6 ± 0.8	−4.2 ± 0.9	−1.5 ± 0.3	0.0 ± 0.04	−0.1 ± 0.05
	Charge–charge [e]	7.2 ± 1.1	5.2 ± 0.3	3.1 ± 0.5	0.2 ± 0.05	0.8 ± 0.04
	Total (low energy) [e]	3.6 ± 0.9	1.0 ± 1.0	1.6 ± 0.6	0.2 ± 0.05	0.7 ± 0.1
	(high energy) [f]	7.9	3.7	1.8	0.2	0.8
	Experiment [g]	0.63	1.41	1.26	0.31	0.67

[a] Values in kcal mol^{-1} at an ionic strength of 0.125 M, expressed as difference between complex and dissociated proteins. E57 and E58 cases are for the optimized salt bridges. PARSE charges were assigned to all residues.
[b] Solvation interaction: change in interaction of designated residue with its own reaction field upon binding.
[c] Charge–charge: change in interaction of designated residue with all other hirudin and thrombin residues upon binding.
[d] Mutant Asn or Gln residue was built with the sidechain amide nitrogen in the OD2/OE2 position (A) or OD1/OE1 position (B) respectively, as indicated.
[e] Differences calculated using the lower energy conformations of the Asn/Gln sidechain, A for D55N, E57Q, B for E58Q, E61Q, E62Q. Numerical error is estimated from the standard deviation of 27 FDPB runs with different molecular positions on the lattice.
[f] Net binding energy change using the higher energy ASN/GLN sidechain orientation. [g] Values taken from Table 2 of Karshikov et al. [10].

Table 6
Electrostatic free energy contributions to binding—effect of multiple site mutations [a]

	E57,58Q	E61,62Q	E57,58,62Q	E57,58,61,62Q
Total contribution				
Calculated [b]	2.9	1.0	3.7	4.0
Experiment [c]	1.42	0.83	2.20	2.50
Non-additivity [d]				
Calculated	0.3	0.0	0.3	0.4
Experiment	−1.25	−0.15	−1.14	−1.15

[a] Values in kcal mol^{-1} at an ionic strength of 0.125 M PARSE charges were assigned to all residues.
[b] Calculated by adding single mutation values in < TBLR > 5 < /TBLR > with appropriate residue–residue interaction terms from Table 2, using < FDR > 7 < /FDR > .
[c] Values taken from Table 2 of Karshikov et al. [10].
[d] Obtained from multiple mutation value minus sum of single mutation values.

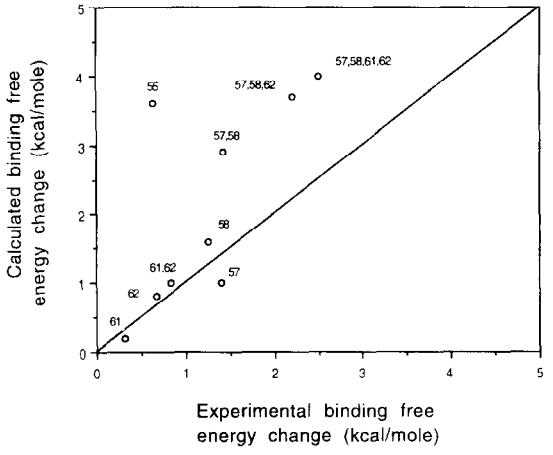

Fig. 2. Comparision of experimental changes in binding free energy, and those calculated using the FDPB method with PARSE parameters, including both solvation and charge–charge interactions. Mutated residue numbers are indicated on the graph. Line of unit slope is indicated on the graph

other residue charges, including the ionized and dipolar charged groups, the contribution of the polar interaction of the mutant Asn/Gln residue, and the possibility of the different Asn/Gln sidechain orientations. The results are uniformly smaller than the formal charge–charge interaction contributions seen in Table 4 because of compensating solvation terms, and because of the residual electrostatic interactions possible in the polar mutant side chains Asn/Gln. Except for D55, the agreement with experiment is probably about as good as one expects from these kind of calculations given the approximations inherent in applying the FDPB method to fixed structures. The results lie within the estimated numerical precision of the FDPB calculations.

The calculated effect of multiple site mutations obtained by combining the single site mutant values of Table 5 with the non-additivity contributions of Table 2, using Eq. (7) (see Section 2 are presented in Table 6. The non-additivity contribution is potentially a measure of the strength of the interaction between two or more residues, and the experimental and calculated non-additivity terms are also given separately in Table 6. In all the cases, the experimental additivity contribution is negative. The calculations fail to reproduce this effect, producing a smaller and, in all cases, positive value. The results of the single site and multiple site calculations are summarized and compared to experimental values in Fig. 2. This clearly illustrates the good agreement with the single site mutations, except for D55, and the uniform overestimate for the multiple site mutations.

Table 7
Effect of lattice contacts on binding

Salt Bridge	Structure	Binding Free energy contribution (kcal mol^{-1})			
		Distance (Å) [b]	Solvation	Charge–charge	Total
R75–E57	Crystal	6.3	−2.4	3.5	1.1
R75–E57	Solution [a]	2.9	−4.2	5.2	1.0
R77A–E58	Crystal	3.5	−3.2	0.2	−3.0 (−1.5) [c]
R77A–E58	Solution [a]	3.2	−1.5	3.1	1.6

[a] With optimized thrombin-to-hirudin salt bridge.
[b] Mean O-to-N distance.
[c] Figure in brackets includes a 1.5 kcal mol^{-1} protonation equilibrium correction.

The effect of the putative salt bridge distortion/exchange induced by crystal lattice contacts is given in Table 7. In the R75–E57 case, the net change in binding upon optimization of the salt bridge is negligible due to almost exact compensation between the solvation and charge–charge terms. For the R77A–E58 salt bridge in the wild type, optimization leads to a greater loss of binding energy upon mutation. For the crystal structure case, the calculated effect of the mutation is negative because there is little charge–charge contribution, while the wild type Glu is considerably desolvated upon binding. However, for exactly the same reason the pK_a is shifted, and this will have a buffering effect on the binding-energy change. Taking account of the protonation equilibrium contribution using Eq. (9), it provides an opposing 1.5 kcal mol^{-1} contribution, which reduces the magnitude of the change. The protonation correction is negligible for the optimized 'solution structure' salt bridge.

4. Discussion

The acidic carboxy terminal portion of hirudin plays an important part in its tight binding to thrombin, as revealed in the crystal structure of the complex [6]. Detailed calculations of the eletrostatic contribution to binding from five acidic residues in this region of hirudin have been performed using the FDPB method, and compared to the experimentally determined change in binding contribution obtained by site directed mutagenesis [5,10]. An attempt was made to include all the major contributions to the electrostatic free energy change due to such mutations, including the charge–charge interaction with both formally charged and dipolar groups in hirudin and thrombin, the solvation interaction of the residue being mutated, the residual electrostatic interaction of the mutant residue, pK_a shifts in the mutated residues, and the effects of ionic strength. The possibility of structural changes was not explored in depth; this can become an open ended procedure in the absence of sufficient experimental and structural data on the hirudin mutant complexes. However the possibility of two amide group orientations in the mutant residue side-chains was considered. In addition examination of the crystal lattice contacts in the hirudin–thrombin structure strongly suggests that the salt bridges Glu58–R77A and Glu57–R75 seen in the structure are much weaker than in solution. The former is distorted by a lattice contact, while the latter is involved in salt bridge partner exchange with the equivalent residues in a neighboring complex around a crystallographic two-fold axis. Conservative reoptimization of the structure of these two salt bridges to represent a plausible solution structure, and its consequence for binding was examined.

The single mutant effects on binding energy were close to the experimental values, except for the D55N mutant whose effect is overestimated. In all cases there is a loss in stability of the complex. The net loss in stability arises from a loss of favorable charge–charge interactions with thrombin, compensated to a significant degree by the less unfavorable desolvation interaction, and by residual polar interactions in the Asn/Gln mutants. The results are considerably better than reported in previous calculations [10]. which did not include the solvation term or Asn/Gln interactions and which consequently significantly overestimated the effect of removing the carboxyl charge. Residues D55, E57, and E58 of hirudin have larger measured contributions to binding, since they are involved in a ladder of salt bridges with R73, R75, R77A of thrombin, while E61 and E62 have less interaction with thrombin, and smaller contributions. The calculations reproduce this trend.

The effect of pK_a shifts upon the binding was negligible except in the E58Q case using the crystal structure. In this case the calculated binding-energy change has the wrong sign. In addition the pK_a is shifted upwards, causing a proton to be taken up upon binding (under physiological pH's), which reduces the effect of the mutation. However the pK_a shift and the gain in binding energy calculated in this case both arise since in the crystal structure it does not make a good salt bridge to R77A because of a lattice hydrogen bond contact. If it is assumed that in solution in the absence of the lattice contact there is an optimal salt bridge, the pK_a shift disappears, and the calculated effect of the mutant has the correct sign. Interestingly the binding is relatively insensitive to the geometry of R75–E57 salt bridge because of the compensation between changes in charge–charge and solvation interactions. This illustrates a

general feature of salt bridge interactions revealed by electrostatic calculations: the subtle balance between desolvation and charge–charge interactions formed upon binding, and their dependence upon structural context [32–34]

The effect of mutation of D55 which is involved in salt bridge interaction with R73 and K149E with thrombin in the crystal structure is significantly overestimated compared to experiment (3.6 kcal mol^{-1} compared to 0.6 kcal mol^{-1}). The effect of this mutation was also greatly overestimated in previous modified Tanford–Kirkwood calculations [10], and this was attributed to structural changes in solution that moved K149E away from R73 to relieve unfavorable interactions, so that the K149–D55 interaction is not present or greatly weakened in the solution binding complex. This effect would reduce the electrostatic destabilization upon D55N mutation. Analysis of the strength of the K149–D55 interaction in the wild type with the FDPB method described here shows about a 1.1 kcal mol^{-1} interaction between these two residues, which if missing in the solution structure would reduce the discrepancy between calculated and experimental values by 1.1 kcal/mole, still leaving an overestimate of 1.9 kcal mol^{-1}. Given the good agreement with the other mutations, it thus seems plausible that the discrepancy in the D55 case is due to structural changes compared to crystal structure in this region, as suggested previously [10]. However this appears to involve more than loss of interaction with the K149 residue of thrombin. Another possible explanation could be competitive effects due to anion binding. The role of chloride in decreasing the hirudin on rate (and hence the binding affinity) has been identified by thermodynamic analysis, and it has been found that one bound chloride in the fibrinogen recognition site is displaced by hirudin binding [9]. If binding of a particular hirudin acidic residue, say D55, to thrombin involves exchange with a negatively charged ion then the effect of that group's charge is less, and so the effect of removing it by mutation would be less. The effect of specific ion binding was not incorporated into the FDPB calculations because the chloride binding-site is not known. However the region of D55 binding on thrombin seems a likely place for this site, since the anion could interact with two basic residues, K149 and R73, and there is a small cleft formed by these residues (see right hand side of Fig. 1).

The difference between the sum of two single mutant binding-energy changes and the corresponding multiple mutant change potentially provides an estimate of the interaction between the two residues in the wild type protein complex, as described previously [28], and described here for purely electrostatic interactions by Eq. (7). All the experimental multiple mutation/single mutation data leads to negative interaction terms (Table 6). This is a rather interesting result. In the wild-type hirudin–thrombin complex the calculated interaction between the acidic residues is more unfavorable than in the unbound hirudin (positive values, Table 2), not surprisingly as they all bear a negative charge, and the screening effect of solvent between these interactions is reduced when bound to thrombin. It is difficult to conceive of any case where an electrostatic interaction of this type would not be positive. The effect of different distances and solvent interaction may change the magnitude of the interaction, but not its sign. So this would imply that the effect of a multiple mutation should be more than the sum of the single mutants, opposite to what is observed resulting in poor agreement for the multiple mutants. (Fig. 2). Given the fact that the single mutant numbers are in reasonable agreement with experiment (except for D55 which is not involved in any of the multiple mutants), the implication of this analysis is that significant structural change must be occurring when two or more of the C-terminal residues are mutated that weakens an unfavorable interaction (or strengthens a favorable interaction which seems less likely) that is present in the wild-type and single-mutant complexes. This argument may be formalized as follows. Assume that in the wild type complex the binding free energy is given by

$$\Delta G^{wt} = \Delta G^{i} + \Delta G^{j} + \Delta G^{ij} + (\Delta G^{p} + \Delta G^{int}) \quad (10)$$

where ΔG^{i} and ΔG^{j} are the contributions of residues i and j, including solvation and charge interactions with the rest of the protein, ΔG^{ij} is the interaction between i and j, and $(\Delta G^{p} + \Delta G^{int})$ is the contribution from the rest of the protein excluding residues i and j, which has been divided up into two parts, the first is assumed to be always present, while the

second is an unfavorable interaction ($\Delta G^{int} > 0$) that is assumed to disappear due to structural changes in the double mutant but is present in the single mutants. Then the change in binding free energy due to single mutations at i or j is given by

$$\Delta\Delta G_i = -\Delta G^i - \Delta G^{ij} \quad (11)$$

$$\Delta\Delta G_j = -\Delta G^j - \Delta G_{ij} \quad (12)$$

The change due to a double mutant is

$$\Delta\Delta G_{ij} = -\Delta G^i - \Delta G^j - \Delta G^{ij} - \Delta G^{int} \quad (13)$$

The difference between the double mutant and the sum of the single mutants is

$$\Delta\Delta G_i j - \Delta\Delta G_i - \Delta\Delta G_j = \Delta G^{ij} - \Delta G^{int} \quad (14)$$

which in the case of ΔG^{int} being zero just gives the i–j interaction (see Eq. (7)). This interaction would be positive from electrostatic interactions alone as discussed above. However if ΔG^{int} is large enough the double mutant/single mutant difference given by Eq. (14) would be negative. For example ΔG^{int} might be an unfavorable steric interaction which is tolerated to promote good salt bridges in the wild-type or single mutants, but is relieved upon removal of two or more salt bridges because the overall binding is weakened. Another mechanism would be electrostatic, in which two positively charges groups on thrombin are brought into close proximity so that they can both interact favourable with the hirudin acidic groups. The positively charged groups would interact unfavorable, unless the acidic groups that bring them together are removed. Examination of the crystal structure in the region of E57 and E58, which show the most interaction shows no obvious candidates for such a conformational change. The effect may also be due to a series of small changes distributed around the mutant site but these issues require detailed knowledge of structural changes of the complex caused by multiple mutants.

An interaction like that between hirudin and thrombin, which involves a number of salt bridges, will in general be sensitive to ionic strength. The hirudin–thrombin binding energy is sensitive to ionic strength, and the sensitivity is affected by the mutations in the acidic C-terminal region of hirudin [5]. However the presence of sodium and chloride binding sites on thrombin complicates the interpretation of these salt sensitivity measurements when the buffer contains sodium. The salt sensitivity was not examined here: The calculations reported here are all done at the same ionic strength, and only the differential effect of different mutations at a single ionic strength are considered.

In summary, agreement between calculated and experimental binding-energy changes for hirudin–thrombin is good for some of the mutations that were examined. The net binding-energy changes result from a balance between charge–charge interactions, solvation, and residual electrostatic interaction in the mutants. In cases where agreement was poor, primarily for the multiple mutants, there is good evidence, both from examination of the crystal structure of the wild type complex, or from analysis of the internal consistency of the calculations that there are structural changes in the mutant. Availability of the mutant structures in the future would allow these structural changes to be incorporated into the FDPB calculations to test whether these structural changes are responsible for the difference in binding energies.

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