Volume 281, number 1,2, 219–222 FEBS 09558 April 1991

ADONIS 001457939100273A

Modification of the stability of phospholipase A2 by charge engineering

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Received 27 November 1990; revised version received 4 February 1991

Electrostatic interactions play an important role in stabilizing the folded conformation of globular proteins. Here we predict the change in stability of charge engineered mutants, construct these mutants and compare the predicted change in stability with that observed. The change in stability was correctly predicted for two of the three mutants and the factors responsible for the discrepancy between observation and prediction for the third mutant are discussed.

Protein engineering of phospholipase A2; Charge engineering; Protein stability

1. INTRODUCTION

Electrostatic interactions, including charge helix-dipole interactions [1,2] and calcium-ion binding [3] have been shown to be important in stabilizing the folded conformation of globular proteins. The stability of a globular protein is the difference in the free energies of the folded and unfolded states and the assumption that underpins this work is that the amino acid substitutions affect the stability of the folded state but do not significantly alter the stability of the unfolded state. This assumption is certainly not valid for substitutions involving proline and glycine residues that have a pronounced effect on chain entropy [4] but is used here to predict the effect of substitutions on protein stability for which we judged that the probable effect on chain entropy is small. Phospholipase A2 is an enzyme of both pharmaceutical and industrial significance and modified stability may have advantages in specific industrial applications. This relatively small enzyme of 124 amino acids catalyses the cleavage of the fatty acid chain at the second position of sn-3 phosphoglycerides. The enzyme is relatively stable (although there has not been a thorough contemporary study of stability) and has 7 disulphide bridges which presumably stabilise the wild type (active) conformation at the potentially denaturing water–phospholipid interface. A calcium ion is essential for the activity of phospholipase A2 (the active site calcium) and a second calcium ion takes part in a contact between molecules in the crystal [5] of the porcine enzyme. This second calcium ion is not found in other mammalian pancreatic enzymes.

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2. EXPERIMENTAL

The effects of charge substitutions on the stability of the porcine enzyme were predicted using the structure of the enzyme as determined by X-ray analysis (protein databank coordinates 1P2P [5]). Amino acid substitutions were modelled using the programme FRODO [6] on an Evans and Sutherland graphics system. The effect of charge changes on the overall electrostatic stability of the protein can be estimated by calculating the electrostatic energy of the modified charge distribution and comparing this electrostatic energy with that of the native charge distribution. The electrostatic interaction energies were calculated as a function of pH and number of calcium ions bound to the protein. The charges of the ionizable side chains of amino acids and charges representing the macro dipole of the α-helices [7] were included in these calculations (see legend of Fig. 1 for more details). Three mutants, each predicted to have modified stability, were selected on the basis of this graphics work and electrostatics calculations. These mutants were then constructed and wild type and mutant protein produced by expression in *E. coli*. The phospholipase A2 gene was subcloned into the M13mp18 phage vector and mutations introduced using the method of Eckstein. The DNA sequence was confirmed by sequence analysis and the cDNA was expressed as a cro-lacZ fusion protein [8] in the plasmid pOK13.2. Active protein was obtained by refolding from inclusion bodies and this active protein was assumed to be correctly folded. The stability of wild-type and mutant proteins was measured by the change in free energy of reversible denaturation of the enzymes. The denaturation was followed by UV-difference spectroscopy. We chose guanadinium hydrochloride (GuHCl)-induced denaturation rather than thermal denaturation because at pH 7.0 GuHCl denaturation as determined by the UV difference spectroscopy can be approximated to a two-state process whereas thermal denaturation cannot (see legend for Fig. 2 for more details).

3. RESULTS AND DISCUSSION

The first two mutations (N89D, N89D/E92K) are close to the N-terminal end of helix 5 (89–108). The third mutant (D42N) is closer to the active site and aspartic acid 42 interacts with the calcium-binding loop (28–32). Asp-42 is on the other side of the calcium-binding loop to the calcium and the mutation on D42N

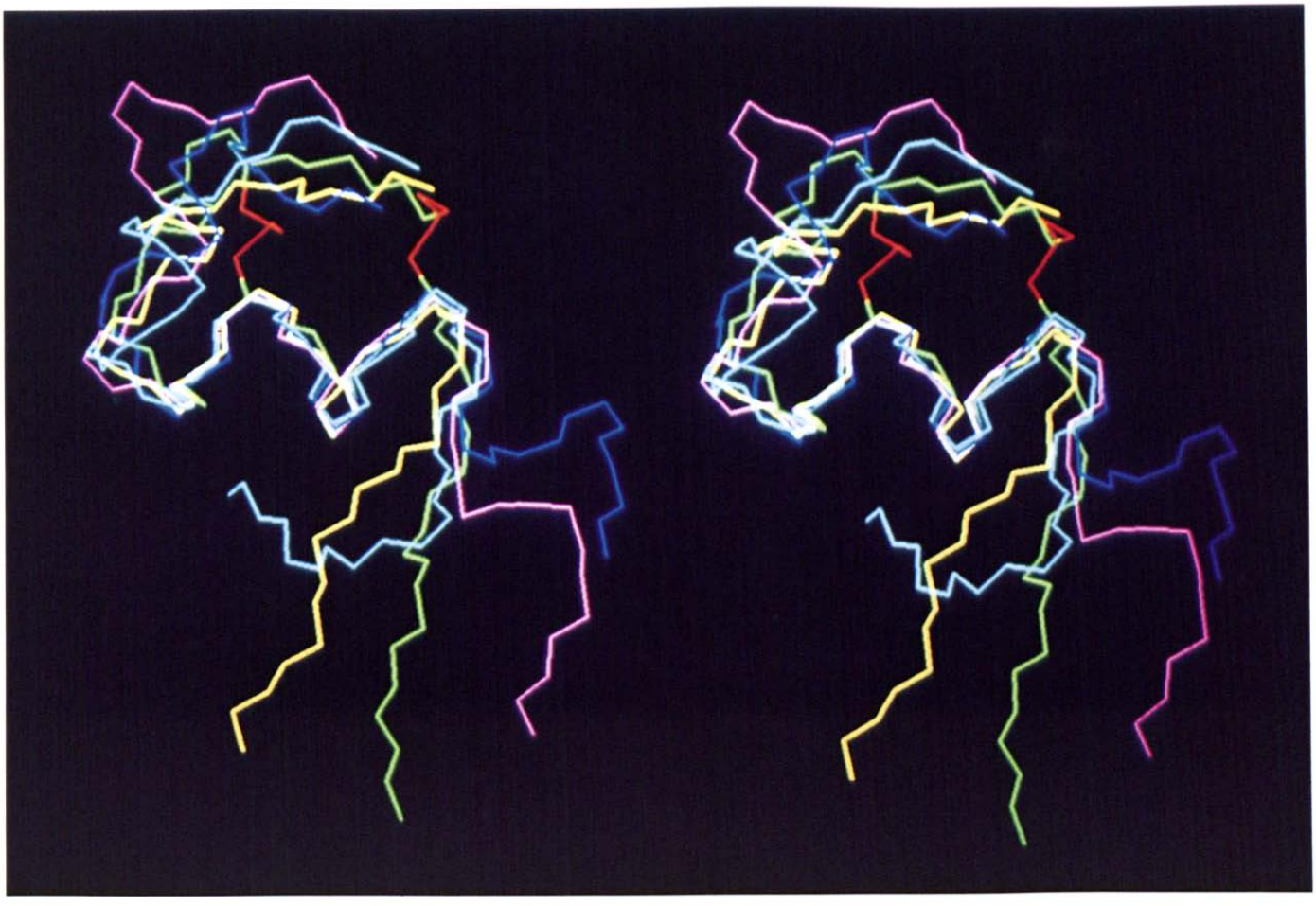

Fig. 1. Asn-89 (pink) at the N-terminal end of an α-helix (yellow ribbon) in native pla2. Glu-92 also shown (pink). The N89D mutant is isosteric and Lys-92 of the N89D/E92K mutant is shown model built in blue. The electrostatic field of the native protein is shown as wire mesh. The blue mesh (+50 mV) is due to the helix macro-dipole and the red mesh (−50 mV) due to Asp-89, Glu-92 and Glu-71. Electrostatic interaction energies (W_{ij}) were calculated using the following charge-charge interaction:

$$W_{ij} = \frac{e^2 Z_i Z_j}{4\pi\epsilon_o\epsilon_{ij} r_{ij}}$$

An effective dielectric (ϵ_{ij}) of 50 and a Debye-Huckel screening factor [9] corresponding to an ionic strength of 0.1 M were used in these calculations. The charges on sites i and j are Z_i and Z_j and are separated by r_{ij}, ϵ_o is the permittivity of free space, and e is the charge on an electron. The difference in overall electrostatic energy between the mutated and wild type enzyme was used to predict the effect of the amino-acid substitutions on the stability.

is expected to reduce the affinity of calcium binding. In the wild-type enzyme asparagine 89 makes hydrogen bonds with the amides of residues 91 and 92 and a substituted aspartic acid (N89D) is expected to make these hydrogen bonds and interact more favourably with the N-terminal end of the helix dipole. However the substituted aspartic acid will interact unfavourably with the adjacent glutamic acid 92 (Fig. 1). Calculation with one calcium ion bound (active site calcium) indicated that at pH 7.0 the N89D mutant would be 1.6 kJ/mol less stable than the wild-type enzyme. The predicted loss of stability is small and depends on the strength of the favourable interaction of the negatively charged aspartic acid with the positive end of the helix dipole (89–108) relative to the unfavourable interaction and with the adjacent negatively charged glutamic acid. In a second mutant asparagine 89 was substituted by an aspartic acid, as in the first mutant, and in addition glutamic acid 92 was replaced by a lysine (N89D/E92K). The lysine was modelled in an extended conformation such that it did not interact specifically with aspartic acid 89 (Fig. 1). The electrostatic energy of this charge distribution was calculated and indicated that at pH 7.0 the N89D/E92K molecule would be 4.2 kJ/mol more stable than the wild-type enzyme. The third mutant D42N was predicted to be 0.3 kJ/mol less stable than wild-type phospholipase A2 (see Table I for a summary of the predicted changes in stability).

The constructed mutant proteins were active against a mixed micellar substrate. GuHCl denaturation indicates that the D42N mutant is less stable, the N89D mutant is slightly more stable and the N89D/E92K mutant considerably more stable than wild type (Fig. 2). The differences in stability for the mutants are +2.2 kJ/mol for D42N, -0.8 kJ/mol for N89D and -3.2 kJ/mol for N89D/E92K.

The sign of the change in stability of the D42N and N89D/E92K mutants is as predicted from electrostatic calculations; however the N89D mutant did not behave as expected (Table I). If the calculations are modified by modelling the loop consisting of residues 59-70 such that it adopts the conformation seen in the bovine structure [11] the N89D mutant is still predicted to be less stable than wild-type enzyme. On the other hand, calcium or positive counter-ion binding would account for the increased stability of the N89D mutant, but such calcium or counter-ion binding remains to be established experimentally. The most probable cause of the discrepancy between prediction and observation for the N89D mutant is the critical balance of charge-charge and charge-helix interactions. If the D89-helix interaction was underestimated or the D89-E92 interaction overestimated in the original prediction then the discrepancy may be explained. This has been the subject of recent calculations using the numerical method of finite differences [12]. This method accounts for the effects of the irregular boundary between low dielectric, counter-ion inaccessible protein and high dielectric, counter-ion accessible solvent. Inclusion of interactions at the spatial resolution of partial charge separation requires careful grid calculations [13]. With a protein dielectric of 3, a solvent dielectric of 80 and a counter-ion concentration of 0.15 M, calculations of the electrostatic stability at residue 89 in the folded state have an estimated error of about 1 kJ/mol. These preliminary calculations do not include models of residue 89 in the unfolded state, thus omitting charge interaction terms in the unfolded state which are expected to be small, and the change in solvation energy for the net charge of mutant N89D, in going from the unfolded to folded state. This solvation energy change is also expected to be small, with a slight destabilization of N89D in the folded state arising from the presence of the globular protein low dielectric. The calculated difference in stability for the N89D mutant is 1.5 kJ/mol less stable than wild-type. This is similar to the result obtained using a uniform dielectric of 50 (Table I). However, it is instructive to look at the contributions of partial charge and ionisable group interactions to the stability. The interaction energies of wild-type (N89) with partial charges and ionisable groups are -13.7

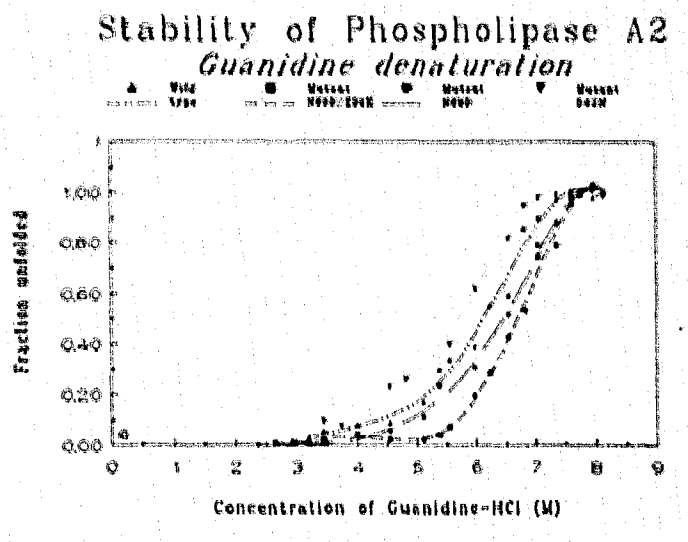

Fig. 2a. Fraction of phospholipase A2 unfolded (f_D) as a function of GuHCl concentration for the wild type (▲), N89D (·), D42N (▼) and N89D/E92K (■) mutants. The fraction folded at any GuHCl concentration is $f_N = 1 - f_D$ and the denaturation constant is given by $K_D = f_D / f_N$.

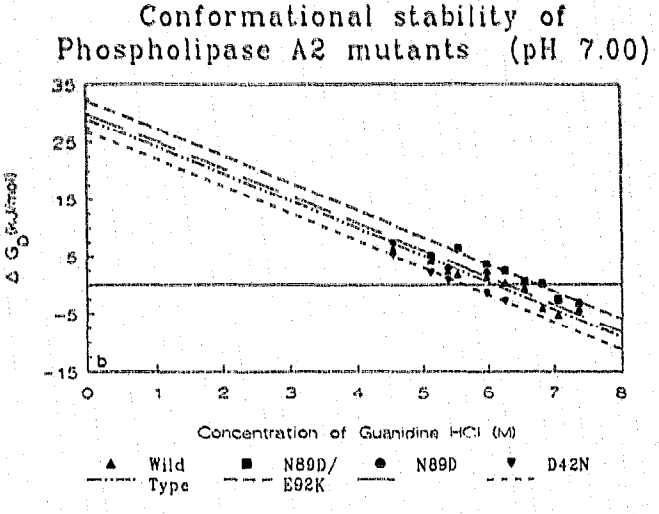

Fig. 2b. The free energy of denaturation (ΔG) is given by $\Delta G_D = -RT \ln K_D$. The free energy of denaturation at zero concentration of GuHCl is estimated by linear extrapolation of the G_D vs. GuHCl plot over the transition region to zero GuHCl concentration [10]. This procedure gives 29.1 ± 0.6 kJ/mol for denaturation of the wild-type enzyme (the values for the mutants are given with respect to the wild-type in Table I)

Table I

Predicted and observed changes in stability of mutant phospholipases A2

Mutant	Change in stability (kJ/mol)	
	Predicted	Observed
N89D	+ 1.6	- 0.8
N89D/E92K	- 4.2	- 3.2
D42N	+ 0.3	+ 2.2

kJ/mol and + 4.0 kJ/mol respectively, and for the N89D mutant these values (partials, ionisables) are -34.8 kJ/mol and + 26.6 kJ/mol respectively. These values give an electrostatic energy for N89 in the wild type folded state of -9.7 kJ/mol, and for the N89D mutant, -8.2 kJ/mol in the folded model. It is seen that the reduced stability calculated as 1.5 kJ/mol is actually due to much larger changes in the partial charge and ionisable group interactions for residue 89. To estimate the effect of coordinate error on the calculated electrostatic energy the carboxy group of E92 was rotated by about 40°. This gave coordinate shifts of about 1.0 Å to each of the oxygen atoms of the E92 side chain whilst preserving a good packing arrangement. This shift reduces the effect of E92 on the 89 site and the N89D mutant is 0.4 kJ/mol more stable than wild-type (with shifted E92). This is in better agreement with experiment (0.8 kJ/mol more stable) and shows that coordinate error or relatively small coordinate shifts in response to this mutation are expected to have a significant effect on the electrostatic energy.

The change in stability of the D42N mutant was greater than predicted. This may be due to diminished calcium-binding affinity which was not taken into account when making the original prediction. Diminished calcium affinity would lead to a greater reduction in stability as observed.

Acknowledgements: We gratefully acknowledge the help of Prof. G. de Haas and Dr. H. Verheij both in giving us the porcine phospholipase A2 DNA clone and also for helpful discussions. We are also pleased to acknowledge EEC funding for one of the authors (J.W.) under the BAP programme (Contract BAP0479UK(H)).

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