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Site-directed mutagenesis and X-ray crystallography of two phospholipase A_2 mutants: Y52F and Y73F

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Tyr52 and Tyr73 are conserved amino acid residues throughout all vertebrate phospholipases A_2. They are part of an extended hydrogen bonding system that links the N-terminal α-NH_3^+-group to the catalytic residues His48 and Asp99. These tyrosines were replaced by phenylalanines in a porcine pancreatic phospholipase A_2 mutant, in which residues 62–66 had been deleted (Δ62–66PLA$_2$). The mutations did not affect the catalytic properties of the enzyme, nor the folding kinetics. The stability against denaturation by guanidine hydrochloride was decreased, however. To analyse how the enzyme compensates for the loss of the tyrosine hydroxyl group, the X-ray structures of the ΔY52F and ΔY73F mutants were determined. After crystallographic refinement the final crystallographic R-factors were 18.1% for the ΔY52F mutant (data between 7 and 2.3 Å resolution) and 19.1% for the ΔY73F mutant (data between 7 and 2.4 Å resolution). No conformational changes occurred in the mutants compared with the Δ62–66PLA$_2$, but an empty cavity formed at the site of the hydroxyl group of the former tyrosine. In both mutants the Asp99 side chain loses one of its hydrogen bonds and this might explain the observed destabilization.

Key words: folding/phospholipase A_2/stability/X-ray structure

Introduction

Phospholipase A_2 (PLA_2) catalyses the hydrolysis of the *sn*-2 ester linkage of phospholipids in a calcium dependent reaction. In the mammalian pancreas the enzyme is produced as a zymogen, which is activated in the duodenal tract by trypsin. The precursor has a similar activity towards monomeric substrates to the mature enzyme, but the activity towards aggregated substrates is considerably higher for the mature enzyme than for the precursor. On the basis of chemical modification studies and crystallographic analysis His48 and Asp99 were identified to be important for catalysis, and Asp49 was shown to be a ligand of the calcium ion in the active centre of the enzyme (Verheij *et al.*, 1980; Dijkstra *et al.*, 1981a). Based on these observations a reaction mechanism was proposed for phospholipase A_2 similar to that of the serine proteases. Instead of a serine residue, a water molecule, activated by the His48/Asp99 pair, acts as the attacking nucleophile to cleave the scissile bond of the substrate. The calcium ion binds the substrate's phosphate group, and also stabilizes the negatively charged transition state (the oxyanion). This mechanism could be confirmed by crystal structures of phospholipases A_2 complexed with a substrate and transition state analogue (Thunnissen *et al.*, 1990a; Scott *et al.*, 1990b).

His48 and Asp99 are linked through an extensive hydrogen bonding network to the N-terminal α-NH_3^+ group. Because modification of the α-NH_3^+ group confers to the mature enzyme precursor-like catalytic properties (Slotboom and de Haas, 1975; Verheij *et al.*, 1981), the hydrogen bonding network has been considered to be essential for catalytic activity (Brunie *et al.*, 1985). Other residues involved in this network are Tyr73 and Tyr52, which make hydrogen bonds with the Asp99 side chain, and a buried water molecule hydrogen bonded to the N-terminal α-NH_3^+ group (see Figure 1). His48, Asp99, Tyr52 and Tyr73 are conserved residues throughout all known sequences of vertebrate phospholipases A_2 (van den Bergh *et al.*, 1989) and their positions in the hydrogen bonding network are superimposable in the different phospholipases (Renetseder *et al.*, 1985). Also with an inhibitor bound in the active site, these residues and the water molecule have the same conformation (Thunnissen *et al.*, 1990a; Scott *et al.*, 1990b). The exact function of the two tyrosine residues has remained unclear, however.

To clarify the role of Tyr52 and Tyr73 site directed mutagenesis experiments have been performed in which these residues were replaced by other amino acids (Dupureur *et al.*, 1990; Kuipers *et al.*, 1990). Substitution by phenylalanines did not significantly change the enzyme's catalytic and binding properties on monomeric and aggregated substrates. This suggests that the hydrogen bonds that these two tyrosines make are not essential for the catalytic activity of phospholipase A_2. If however, the aromatic rings are removed by mutating the tyrosines into Ala, Val, Ser (Dupureur *et al.*, 1990) or Gln (Kuipers *et al.*, 1990) the activity is significantly decreased, although the Gln and Ser mutations still have, in theory, hydrogen bonding capabilities. Dupureur *et al.* (1990) suggested that the hydrogen bonds that are lost by the mutation of Tyr into Phe may be compensated

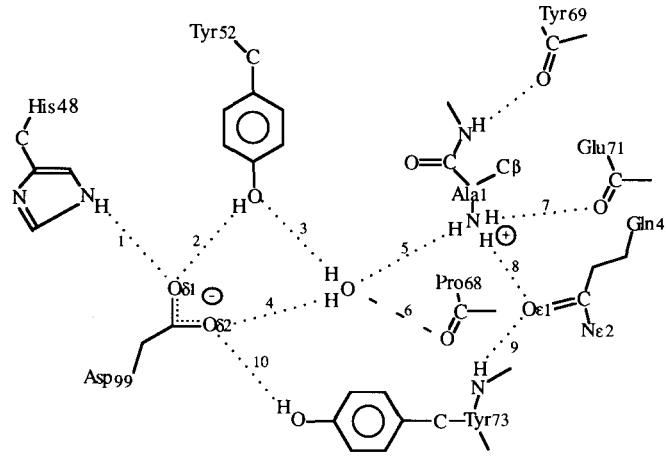

Fig. 1. Schematic drawing of the hydrogen bonding system in the Δ62–66 mutant of porcine pancreatic phospholipase A_2 (Thunnissen *et al.*, 1990b). The hydrogen bonds are numbered according to Table IV.

for by an extra water molecule, or alternatively other residues might take over the function of the hydroxyl group.

Here we report a structural analysis of two porcine phospholipase A_2 mutants, in which Tyr52 and Tyr73 were changed into a phenylalanine (ΔY52F and ΔY73F). The mutations were introduced into a porcine pancreatic phospholipase A_2 mutant in which residues 62–66 in a surface loop were deleted (Kuipers *et al.*, 1990), because of better crystallization properties. This mutant (Δ62–66PLA_2) shows increased activity compared with wild-type porcine PLA_2 (Kuipers *et al.*, 1989; Thunnissen *et al.*, 1990b). The ΔY52F and ΔY73F mutants were further analysed with respect to their folding kinetics and their stability in the presence of guanidine hydrochloride. It appeared that the three-dimensional structure and the folding kinetics were hardly affected by the mutations, but that the substitutions influenced the stability of the enzyme.

Materials and methods

Proteins and refolding

Wild-type porcine pancreatic PLA_2 and the mutants Δ62–66PLA_2, ΔY52F and ΔY73F were obtained as described before (Nieuwenhuizen *et al.*, 1974; Kuipers *et al.*, 1990). S-sulphonation of (mutant) phospholipase was carried out according to Thannhauser and Scheraga (1985) at a concentration of 6 mg/ml of phospholipase. The resulting protein was desalted by dialysis against 1 mM acetic acid. Refolding was carried out by diluting a sample of S-sulpho-(mutant) phospholipase to a final concentration of 75 μg/ml in buffer containing 2 M urea, 5 mM EDTA, 5 mM borate, 8 mM cysteine and 1 mM cystine at pH 8.3. The refolding was monitored by testing aliquots for reappearance of enzymatic activity in the egg yolk phospholipase assay (Nieuwenhuizen *et al.*, 1974).

Determination of free energy of denaturation

Denaturation of PLA_2 was carried out essentially as reported before (Pickersgill *et al.*, 1991), but because of the greater sensitivity the unfolding was followed by the increase of tyrosine fluorescence rather than by ultraviolet spectroscopy. Guanidine–HCl (Merck) was recrystallized according to Nozaki (1972). PLA_2 (final concentration 7 μM) was dissolved in 50 mM PIPES (1,4-piperazine-*NN'*-bis-2-ethanesulphonic acid) pH 7.0 or 50 mM formiate, pH 3.0 in the presence of 2 mM EDTA and varying amounts of guanidine hydrochloride ranging from 0 to 8.25 M. Spectra were recorded at 20°C with a Perkin-Elmer LS-5 Luminescence Spectrophotometer. Excitation was performed at 275 nm and the emission was recorded at 305 nm; slit widths were 5 nm. The free energy of denaturation was determined from the equation $\Delta G_d = -RT \ln K_d$, in which K_d is the equilibrium constant for denaturation. K_d at various guanidine hydrochloride concentrations was determined using the equation $K_d = F_n - F_{obs}/F_{obs} - F_d$, where F_n, F_{obs} and F_d are the fluorescence quantum yields of the native protein, the observed fluorescence and the fluorescence quantum yield of the fully unfolded protein, respectively. At pH 7 the Δ62–66PLA_2 was not fully unfolded at the highest guanidine concentrations and the value of F_d was obtained by extrapolation from the plots of the other PLA_2s. The $\Delta\Delta G_d$ values between wild-type and mutant PLA_2 were calculated using the equation: $\Delta\Delta G_d = m\ \Delta[\text{guanidine}]_{1/2}$, in which m is the slope of the linear denaturation plot of wild-type PLA_2 ($d\Delta G_d/d[\text{guanidine}]$) and $\Delta[\text{guanidine}]_{1/2}$ the difference between wild-type PLA_2 and mutant in guanidine concentration at the midpoint of unfolding (Pace, 1986).

Crystallization

For crystallization each mutant was dissolved in 0.1 M acetate buffer, pH 4.5, to a final concentration of 10 mg/ml. The solution of ΔY52F was dialysed overnight against 100 mM BES buffer [*N,N*-bis(2-hydroxyethyl-)2-aminoethane sulphonic acid], pH 7.0 with 5 mM $CaCl_2$ added; the ΔY73F mutant was dialysed overnight against 100 mM Tris–HCl buffer [tris (hydroxymethyl)aminomethane], pH 7.5 also with 5 mM $CaCl_2$ added. The enzymes were crystallized at room temperature by vapour diffusion in hanging drops. The reservoir solution for each mutant contained 50% methanol in their respective buffer. In each case drops of 6 μl were formed by equal mixing of 3 μl protein solution and 3 μl of the reservoir solution. Crystals for both mutants, suitable for X-ray analysis, grew within 1–3 weeks. The crystals are platelets of ~0.4 mm * 0.4 mm * 0.2 mm–0.7 mm * 0.3 mm * 0.2 mm in size. The space group for crystals of both mutants is $P2_1$ with cell dimensions of a = 45.7 Å, b = 73.1 Å, c = 37.1 Å and β = 107.4° for the ΔY52F mutant and a = 45.6 Å, b = 73.6 Å, c = 37.1 Å and β = 107.4° for the ΔY73F mutant. There are two molecules of each 119 residues in the asymmetric unit. The cell dimensions of the crystals are only slightly different from those of the crystals of Δ62–66PLA_2 (Thunnissen *et al.*, 1990b) which are a = 45.8 Å, b = 73.4 Å, c = 37.3 Å and β = 107.4°. The crystals diffract to ~2.4 Å resolution.

Data collection

X-ray data for both mutants were collected from a single crystal on an Enraf–Nonius FAST Area detector, equipped with a

Table I. Data collection statistics

Mutant	ΔY52F	ΔY73F
a (Å)	45.70	45.64
b (Å)	73.09	73.58
c (Å)	37.11	37.11
β (°)	107.36	107.40
Resolution (Å)	2.3	2.4
Number of observed reflections	16508	13928
Unique reflections	10701	8361
R-merge (%)	4.32	3.80
Completeness (%)	89.7	88.8
Completeness last shell (%)	59.0	48.8
Last shell (Å)	2.36–2.30	2.43–2.40

$$\text{R-merge} = \frac{\sum_{hkl}\sum_{\text{refl.}} |\, I(hkl,j) - \bar{I}(hkl)\,|}{\sum_{hkl}\sum_{\text{refl.}} |\, I(hkl,j)\,|} \times 100\%$$

Table II. Refinement and geometry statistics

	ΔY52F	ΔY73F
R-factor (%)	18.1	19.1
No. of water molecules	140	86
r.m.s. bond length deviations (Å)	0.011	0.011
r.m.s. bond angle deviations (°)	2.882	2.824
r.m.s. trignonal non planarity deviations (Å)	0.007	0.010
r.m.s. planarity deviations (Å)	0.011	0.011
r.m.s. non bonded interactions deviations (Å)	0.060	0.062

$$\text{R-factor} = \frac{\sum \|\,|F_o| - |F_c|\,\|}{\sum |F_o|} \times 100\%$$

CAD4 kappa goniostat with graphite monochromatized CuKα radiation from an Elliot GX-21 rotating anode generator. Data collection and reduction were carried out using the program system MADNES (Messerschmidt and Pflugrath, 1987). Data were profile fitted with the XDS program (Kabsch, 1988). 180° of oscillation were collected in one setting for both crystals. Statistics for these data are shown in Table I. The data were scaled (Hamilton *et al.*, 1965) and merged using programs of the Groningen BIOMOL protein software package.

Crystallographic refinement

The Δ62−66 phospholipase A_2 without water molecules was used as a starting model in both cases. The mutated residues were checked in F_o-F_c maps. In both cases a negative peak was observed at the position of the OH of the former tyrosines. These tyrosines were manually changed into phenylalanines. Both mutants were refined using the TNT package in combination with model building (Tronrud *et al.*, 1987b). The ΔY52F mutant was refined using all data between 10 and 2.3 Å resolution, the ΔY73F mutant with data between 10 and 2.4 Å resolution. During the refinement the model was checked using $2F_o-F_c$ maps with SIGMAA coefficients (Read, 1986) and OMITmaps (Bhat, 1988) on an Evans and Sutherland PS390 picture system using the program FRODO (Jones, 1985). Water molecules were located as peaks in F_o-F_c maps at proper distances from polar atoms using the PEKPIK option of the XTAL package (Hall and

Table III. Kinetic properties of phospholipase A_2 mutants (from Kuipers *et al.*, 1990)

	Affinities for substrate analogues		Enzymatic activities on monomers and micelles			
	Monomeric C12PN K_d (mM)	Micellar C16PN $N \cdot K_d$ (μM)	DiC6dithioPC (monomers) k_{cat}/K_m ($s^{-1}M^{-1}$) × 10^{-3}	DiC8PC (micelles) V_{max} (mmol/min/mg)	DiC8PC (micelles) K_m (mM)	DiC8PC (micelles) k_{cat}/K_m (s^{-1} M^{-1})×10^{-3}
Δ62−66	0.8	140	1.64	4310	2.8	366
ΔY52F	0.8	172	1.61	4750	2.5	452
ΔY73F	0.8	118	1.31	4360	2.0	519

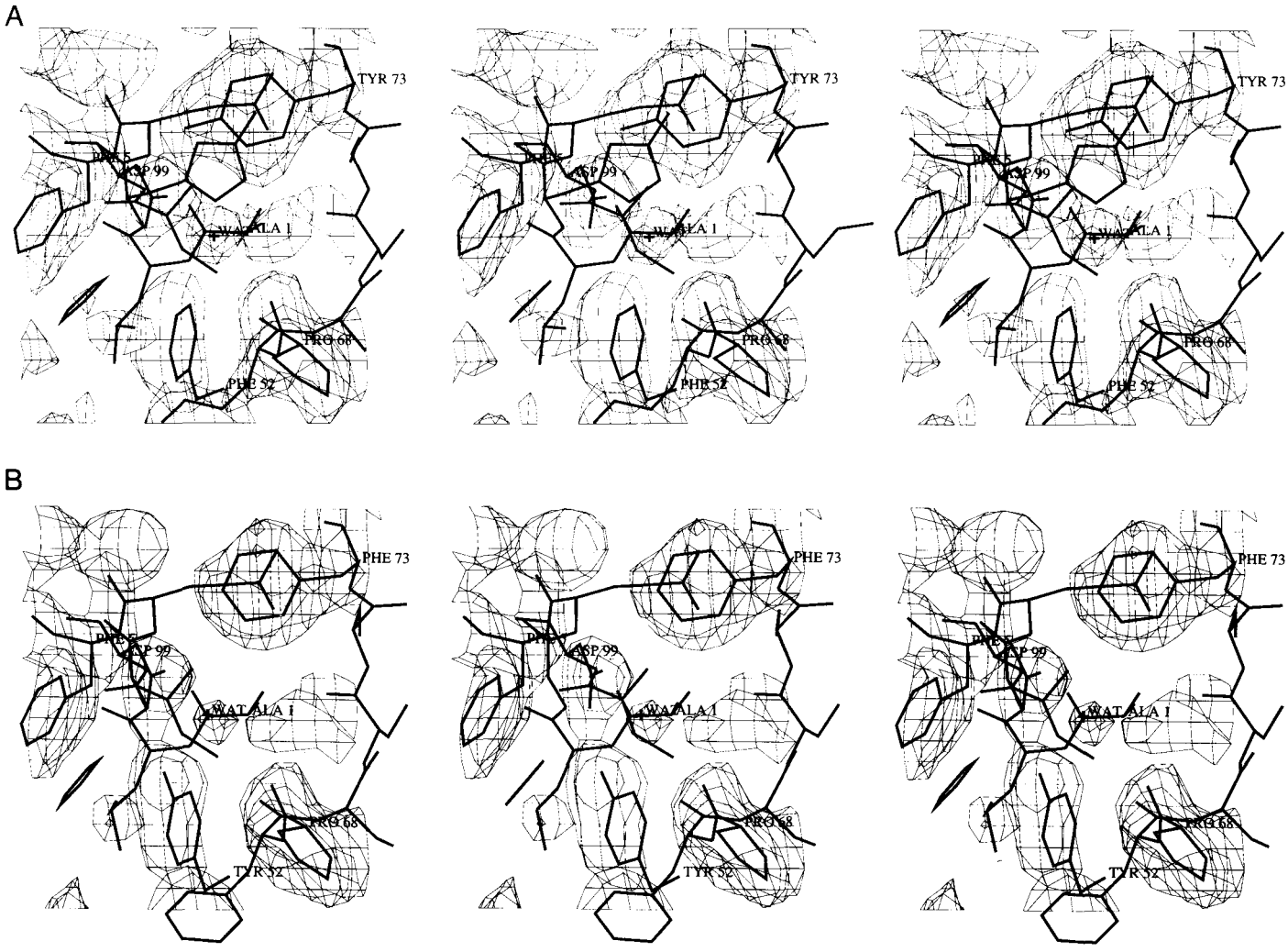

Fig. 2. Stereopictures showing ($2mFo-DFc$),α_{calc} electron density (Read, 1986) contoured at 1.1 σ. (**A**) Surroundings of residue 52 in the ΔY52F mutant. (**B**) Surroundings of residue 73 in the ΔY73F mutant.

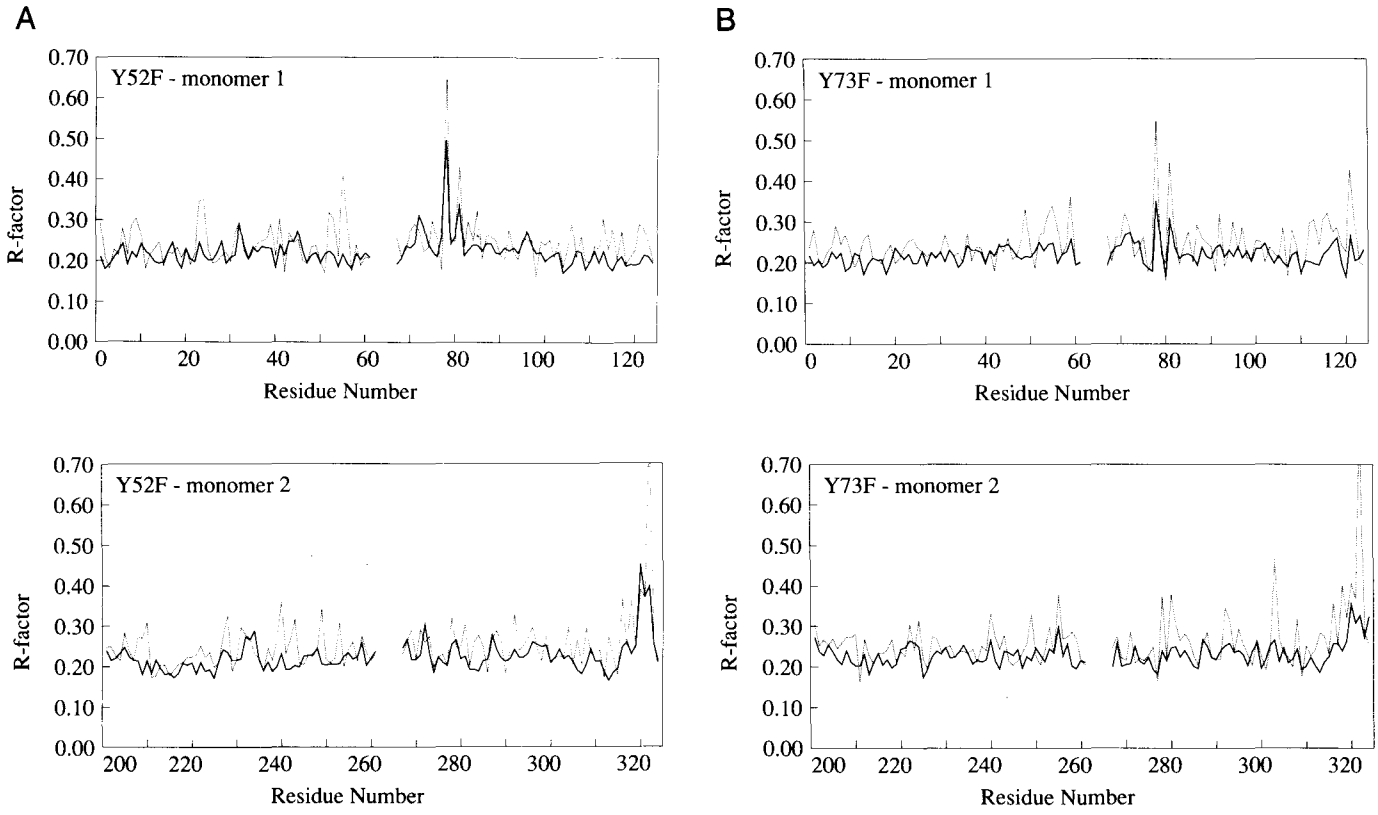

Fig. 3. Real space R-factor per residue (Jones *et al.*, 1991). Solid lines are for the main chain atoms, dotted lines for side chain atoms. (**A**) Monomer 1 (residues 1–124) and monomer 2 (residues 201–324) of the ΔY52F mutant. (**B**) Monomer 1 (residues 1–124) and monomer 2 (residues 201–324) of the ΔY73F mutant.

Stewart, 1987). The quality of the models was checked during the refinement by Ramachandran plots, the NEWGEO and DSCREEN modules of TNT and the QUALTY option of the WHATIF program (Vriend, 1990). More details of the refinement can be found in Table II.

Results

Catalytic properties

As has been shown before, the catalytic properties and the binding affinities of the ΔY52F and ΔY73F mutants towards both monomeric and aggregated substrates are not very much affected compared with Δ62–66PLA$_2$ (Kuipers *et al.*, 1990). For reference we give a summary of these properties in Table III.

Three-dimensional structures

To verify also that the three-dimensional structures of ΔY52F and ΔY73F are similar to that of Δ62–66PLA$_2$, we determined their crystal structures. Because they crystallize isomorphously with the Δ62–66PLA$_2$, their structures could be determined by direct crystallographic refinement of the Δ62–66PLA$_2$ model against the observed structure factors of the mutants. This refinement was straightforward for both mutants and no major rebuilding was necessary. The electron density for both mutated residues was well defined (Figure 2). The final crystallographic R-factors ($\Sigma|F_o-F_c|/\Sigma F_o$) are 18.1% and 19.1% for the ΔY52F and ΔY73F mutants respectively. Table II gives additional refinement and structural details of the two structures. As in the Δ62–66 mutant, part of the C-terminus of the second monomer is in very poor density (Thunnissen *et al.*, 1990b). Also the residues in the β-wing tip (residues 78–81) are not very well defined. This is reflected in Ramachandran plots (Ramakrishnan and Ramachandran, 1965), where a few residues occur outside the allowed regions (plots not shown). These residues are Thr80 (ϕ = 94°, ψ = −39°) for the Y52F mutant and Thr80 (ϕ = 90°, ψ = −17°), Thr 280 (ϕ = 89°, ψ = −23°) and Lys322 (ϕ = 172°, ψ = 3°) for the Y73F mutant. A per residue fit of the model to the electron density can be seen in a real space R-factor plot (Figure 3; Jones *et al.*, 1991).

No major conformational changes have occurred in either one of the mutants in comparison with the Δ62–66 mutant. The root-mean-square (r.m.s.) differences in the 119 Cα positions are 0.30 and 0.32 Å for monomers 1 and 2, respectively of the ΔY52F mutant. For the Y73F mutant the r.m.s. differences are 0.26 and 0.24 Å for monomers 1 and 2. The catalytic residues (His48 and Asp99) and the residues that are part of the hydrogen bonding system (Ala1, Gln4, Tyr/Phe52, Pro68, Glu71 and Tyr/Phe73) do not change in position within the limit of error (r.m.s. differences 0.23–0.26 Å) and also the water molecule that connects the N-terminal NH_3^+ group with the catalytic residues His48 and Asp99 is within 0.25 Å from its position in Δ62–66PLA$_2$ in both mutants (Figure 4; Table IV). In each structure this water molecule loses one of its hydrogen bonds. No density, in either the ΔY52F or ΔY73F mutant, can be observed for an extra water molecule near the deleted hydroxyl group of the mutated tyrosines. In addition, no significant movement of amino acid residues towards the space left by the deletion of the hydroxyl groups of the tyrosines can be observed in either case (Figure 4). Therefore, we conclude that replacement of either Tyr52 or Tyr73 by a Phe does not affect the three-dimensional structure, and that Tyr52 and Tyr73 are not essential for catalytic activity.

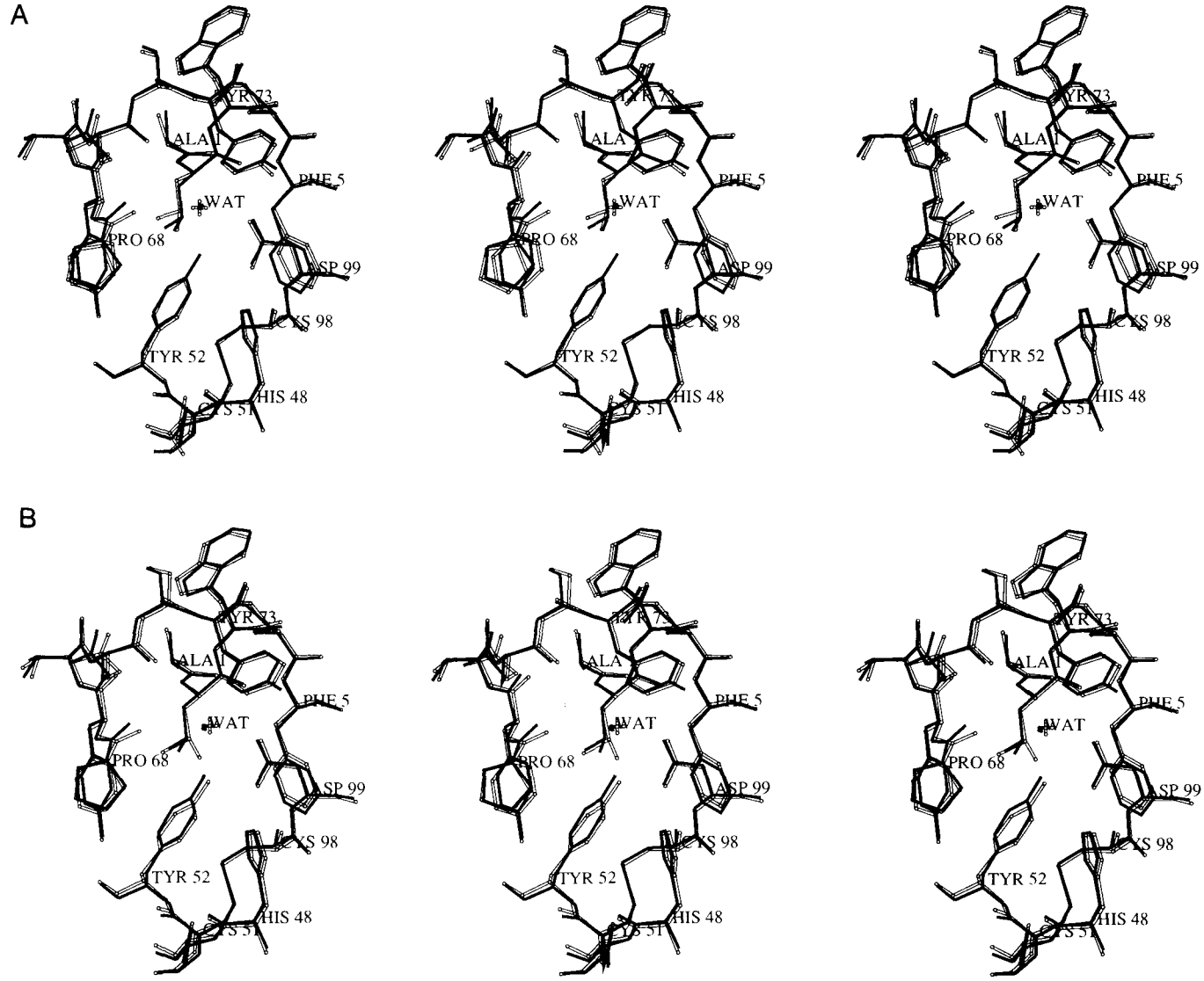

Fig. 4. Stereopictures showing superpositions of the active sites of the ΔY52F and ΔY73F mutants with the parent Δ62−66 porcine pancreatic phospholipase A_2. (**A**) Active sites of ΔY52F (bold lines) and Δ62−66 (open lines). (**B**) Active sites of ΔY73F (bold lines) and Δ62−66 (open lines).

Folding kinetics

While the conservation of Tyr52 and Tyr73 is apparently not important for the three-dimensional structure, these residues could be essential for the folding kinetics of the enzyme. This possibility was tested by following the rate of reoxidation/refolding of phospholipase A_2 in which all 14 cysteine residues had been converted into S-sulphocysteine. Refolding (as judged by the re-appearance of enzymatic activity) is remarkably rapid for a protein in which seven disulphide bridges have to be formed. At room remperature the half-time of refolding is 105 ± 15 min and this value is 29 ± 6 min at 37°C for native porcine pancreatic PLA_2 and for the Δ62−66PLA_2, ΔY52F and ΔY73F mutants. Thus the mutations induce little or no change in the refolding rate of phospholipase A_2.

Stability

A third reason for the conservation of Tyr52 and Tyr73 might be that these residues are important for the stability of phospholipase A_2. Therefore, we determined the free energy of denaturation ΔG_d, from the concentration dependence of the equilibrium constant, K_d. For wild-type PLA_2 the free energy of denaturation at zero concentration guanidine (ΔG_d^0) was obtained by linear extrapolation and appeared to be 30.4 kJ/M. This value is in good agreement with the value (29.1 kJ/M) reported by Pickersgill *et al.* (1991) who followed unfolding of PLA_2 with guanidine by ultraviolet absorbance difference spectroscopy instead of fluorescence spectroscopy. The free energy of denaturation as a function of the guanidine concentration of wild-type and mutant PLA_2s is shown in Figure 5. The guanidine concentration at the midpoint of denaturation of wild-type PLA_2 and of the mutants is shown in Table V. From these data it can be concluded that the deletion of residues 62−66 in Δ62−66PLA_2 has a stabilizing effect. The subsequent substitution of Phe for Tyr at position 52 or 73 then has a destabilizing effect, be it that these mutants are still more stable than the wild-type PLA_2. The simultaneous introduction of two phenylalanines at positions 52 and 73, however, makes the mutant less stable than the wild-type PLA_2. Because it is known that porcine pancreatic PLA_2 is more sensitive for denaturation at lower pH values (Abita *et al.*, 1972) the denaturation by guanidine was repeated at pH 3.0. Indeed, at this pH value all PLA_2s, including Δ62−66PLA_2 could be denatured completely. The

Table IV. Hydrogen bond geometries in the N-terminal hydrogen bonding system

H-bond	Donor	Acceptor	B-factor donor (Å^2)	B-factor acceptor (Å^2)	Distance (Å)	H-bond angle (°)
(a) *Δ62–66 mutant*						
1	Nε2 His48	Oδ2 Asp99	19.0	20.2	2.8	Cγ-Oδ2-Nε2 145
2	OH Tyr52	Oδ2 Asp99	14.0	20.2	2.9	Cγ-Oδ2-OH 99
3	H_2O	OH Tyr52	20.8	14.0	2.6	Cη-OH-H_2O 132
4	H_2O	Oδ1 Asp99	20.8	20.2	2.7	Cγ-Oδ1-H_2O 129
5	NH_3^+ Ala1	H_2O	18.7	20.8	3.2	Cα-NH_3^+-H_2O 95
6	H_2O	O Pro68	20.8	23.0	3.5	C-O-H_2O 133
7	NH_3^+ Ala1	O Glu71	18.7	22.4	2.3	C-O-NH_3^+ 143
8	NH_3^+ Ala1	Oε1 Gln4	18.7	22.4	3.2	Cδ-Oε1-NH_3^+ 140
9	N Tyr73	Oε1 Gln4	19.2	22.4	3.0	Cδ-Oε1-N 118
10	OH Tyr73	Oδ1 Asp99	17.9	20.2	2.8	Cγ-Oδ1-OH 108
(b) *ΔY52F mutant*						
1	Nε2 His48	Oδ2 Asp99	15.3	14.9	2.6	Cγ-Oδ2-Nε2 145
2	–	Oδ2 Asp99	–	14.9	–	–
3	H_2O	–	28.2	–	–	–
4	H_2O	Oδ1 Asp99	28.2	15.6	2.6	Cγ-Oδ1-H_2O 135
5	NH_3^+ Ala1	H_2O	15.1	28.2	3.1	Cα-NH_3^+-H_2O 95
6	H_2O	O Pro68	28.2	17.2	3.2	C-O-H_2O 153
7	NH_3^+ Ala1	O Glu71	15.1	13.9	2.5	C-O-NH_3^+ 117
8	NH_3^+ Ala1	Oε1 Gln4	15.1	14.2	2.9	Cδ-Oε1-NH_3^+ 143
9	N Tyr73	Oε1 Gln4	11.2	14.2	3.2	Cδ-Oε1-N 112
10	OH Tyr73	Oδ1 Asp99	17.2	15.6	2.7	Cγ-Oδ1-OH 117
(c) *ΔY73F mutant*						
1	Nε2 His48	Oδ2 Asp99	9.9	13.9	2.8	Cγ-Oδ2-Nε2 145
2	OH Tyr52	Oδ2 Asp99	8.4	13.9	2.8	Cγ-Oδ2-OH 99
3	H_2O	OH Tyr52	32.1	8.4	3.0	Cη-OH-H_2O 132
4	H_2O	Oδ1 Asp99	32.1	12.3	2.6	Cγ-Oδ1-H_2O 129
5	NH_3^+ Ala1	H_2O	15.4	32.1	3.3	Cα-NH_3^+-H_2O 95
6	H_2O	O Pro68	32.1	24.6	3.4	C-O-H_2O 133
7	NH_3^+ Ala1	O Glu71	15.4	16.5	2.4	C-O-NH_3^+ 143
8	NH_3^+ Ala1	Oε1 Gln4	15.4	18.9	3.0	Cδ-Oε1-NH_3^+ 140
9	N Tyr73	Oε1 Gln4	11.7	18.9	3.2	Cδ-Oε1-N 118
10	–	Oδ1 Asp99	–	12.3	–	–

midpoint of denaturation of wild-type PLA_2 was shifted from 6.3 M at pH 7.0 towards 5.25 M guanidine at pH 3.0; the midpoints of denaturation of the mutant PLA_2s shifted similarly (data not shown), confirming the differences in stability that exist at pH 7.0.

Discussion

Within the limits of error the three-dimensional structures of the ΔY52F and ΔY73F mutants show no differences with the Δ62–66PLA_2 mutant. Also the catalytic properties of these mutants are similar to those of Δ62–66PLA_2. From this it can be concluded that Tyr52 and Tyr73 are not essential for the enzyme's catalytic activity or its conformation. Bee venom phospholipase A_2, which shows a low sequence homology (Kuchler *et al.*, 1989) and which is structurally distinct from the mammalian and snake venom PLA_2s (Scott *et al.*, 1990a), lacks also Tyr52 and Tyr73. Although the catalytic His48 and Asp99 are conserved, as well as the residues in the calcium binding loop, Tyr52 and Tyr73 have been replaced in bee venom PLA_2 by one single tyrosine, Tyr87, which is a non-analogous position in the sequence. Thus different solutions for the hydrogen bonding system seem possible without a deleterious effect on the catalytic activity.

The loss of the hydrogen bonding capacities of the hydroxyl groups of Tyr52 and Tyr73 does not affect the folding rate of phospholipase A_2 *in vitro*. While the formation of stable hydrogen bonds in secondary structure elements like α-helices probably occurs somewhere in the early stages of the folding process, the hydrogen bonds that connect the secondary structure elements into the final native tertiary structure are formed later

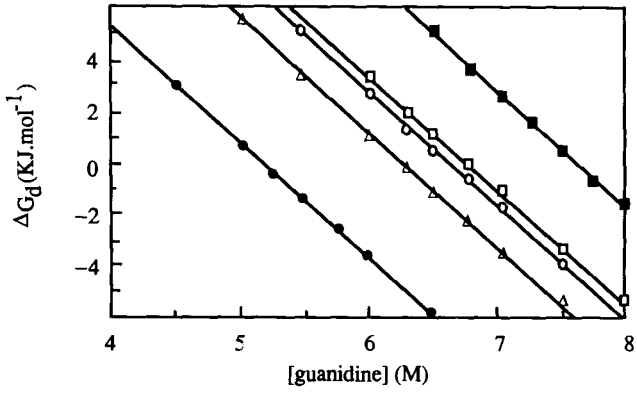

Fig. 5. Δ*G* of unfolding by guanidine hydrochloride of porcine pancreatic wild-type and mutant phospholipases A_2 at pH 7.0. △, wild-type phospholipase A_2; □, ΔY52F; ○, ΔY73F; ■, Δ62–66 phospholipase A_2; ●, ΔY52F+ΔY52F.

Table V. Guanidine–HCl induced unfolding of porcine pancreatic wild-type and mutant phospholipases A_2 at pH 7.0

PLA_2	$[guanidine]_{1/2}$ (M)	$\Delta\Delta G_d$ with respect to wild-type (kJ/mol)	$\Delta\Delta G_d$ with respect to Δ-PLA_2 (kJ/mol)
Wild-type	6.3	–	–
Δ-PLA_2	7.6	+ 6.5	–
ΔY52F	6.75	+ 2.2	– 4.3
ΔY73F	6.65	+ 1.9	– 4.6
ΔY52F,Y73F	5.15	– 6.5	–12.1

$[guanidine]_{1/2}$ is the guanidine–HCl concentration at 50% unfolding

(Matthews, 1991). Although the hydrogen bonds that the Asp99 side chain makes, could belong to this latter class, it is clear that the hydroxyl groups of tyrosines 52 and 73 are not essential for the folding kinetics of phospholipase A_2.

In contrast, the enzyme's stability towards denaturing conditions is clearly affected by the substitutions. When mutating the tyrosines into phenylalanines one hydrogen bond is lost in each mutant: either the hydrogen bond between the Oδ2 atom of Asp99 and the Tyr52 OH or the one between the Oδ1 of Asp99 and the OH of Tyr73 (see Figure 1 and Table IV). The loss of only one of the four hydrogen bonds of the charged side chain of Asp99 evidently does not induce a conformational change in the protein molecule, but it could be the cause of the destabilization of the structure. Hydrogen bonds between uncharged donors and acceptors contribute 2–7 kJ/mol to the stability of a protein, whereas hydrogen bonds involving charged residues may be considerably more effective in stabilizing the protein (Fersht *et al.*, 1985; Fersht, 1987; Tronrud *et al.*, 1987a). The values observed by us for the Tyr/Phe substitutions fall well within this range. A second reason for the decreased stability might be due to the cavity created by mutating the tyrosines into a phenylalanine. Cavities in a protein molecule are energetically unfavourable because their surrounding residues have less van der Waals interactions than if these residues were located in a more densely packed environment. Indeed, replacement of hydrophobic core residues by smaller ones resulted in less stable proteins (Yutani *et al.*, 1987; Kellis *et al.*, 1988; Sandberg and Terwilliger, 1989; Shortle *et al.*, 1990). The empty space, created by the deletion of the hydroxyl groups is not large enough to be filled by a water molecule as is evident from our structures, nor is this space taken up by other residues from the protein molecule, in contrast to the suggestion by Dupureur *et al.* (1990). Recently a much more drastic experiment was reported in which Tyr73 in bovine phospholipase A_2 was replaced by a Ser (Dupureur *et al.*, 1992), thus creating a much larger cavity. This resulted in a highly flexible conformation of phospholipase A_2, in which nevertheless the elements of secondary structure were still preserved. The more subtle Tyr73Phe substitution which we analysed did not give any indication of increase of flexibility. In this respect it should be mentioned that Tyr52 and Tyr73 are involved in perpendicular aromatic–aromatic interactions with His48, Tyr69 and Tyr75 (Dijkstra *et al.*, 1981b). Furthermore, the aromaticity of the phenyl rings might provide a weak hydrogen bond acceptor function (Thomas *et al.*, 1980; Levitt and Perutz, 1988). Therefore, the presence of an aromatic residue at positions 52 and 73 might well be an important factor for the stability of the protein, as has been observed elsewhere (Burley and Petsko, 1985).

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