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Original Citation:

Properties of N-terminus truncated and C-terminus mutated muscle acylphosphatases / N. TADDEI; A. MODESTI; M. BUCCIANINI; M. STEFANI; F. MAGHERINI; M. VECCHI; G. RAUGEI; G. RAMPONI. - In: FEBS LETTERS. - ISSN 0014-5793. - STAMPA. - 362:(1995), pp. 175-179.

Availability:

This version is available at: 2158/1350 since: 2021-02-08T16:47:31Z

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Properties of N-terminus truncated and C-terminus mutated muscle acylphosphatases

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Received 23 January 1995

Abstract Enzymatic activity and structure of N-terminus truncated and C-terminus substituted muscle acylphosphatase mutants were investigated by kinetic studies under different conditions and ^1H NMR spectroscopy, respectively. The N-terminus truncated mutant lacked the first six residues ($\Delta 6$), whereas arginine 97 and tyrosine 98 were replaced by glutamine giving two C-terminus substituted mutants (R97Q and Y98Q, respectively). All acylphosphatase forms were obtained by modifications of a synthetic gene coding for the human muscle enzyme which was expressed in *E. coli*. The $\Delta 6$ deletion mutant elicited a reduced specific activity and a native-like structure. The kinetic and structural properties of R97Q and Y98Q mutants indicate a possible role of Arg-97 in the stabilisation of the active site correct conformation, most likely via back-bone and side chain interactions with Arg-23, the residue involved in phosphate binding by the enzyme. This study also suggests a possible involvement of Tyr-98 in the stabilisation of the acylphosphatase overall structure.

Key words: Acylphosphatase deletion mutant;
Acylphosphatase recombinant; Acylphosphatase mutant;
Acylphosphatase ^1H NMR spectrum

1. Introduction

Acylphosphatase (acylphosphate phosphomonoesterase, EC 3.6.1.7.) is a basic enzyme which catalyses the hydrolysis of acylphosphates both synthetic, such as benzoylphosphate [1], and of physiological interest, such as acetylphosphate, carbamoylphosphate, succinylphosphate, 1,3-diphosphoglycerate, and the phosphorylated intermediate formed during the activity of membrane pump ATPases [2–7]. Acylphosphatase is widely distributed in vertebrate tissues and organs as two isoenzymes displaying about 55% amino acid sequence homology. The genes for the two isoenzymes are probably originated by duplication and evolution of a common ancestor [8]. The two isoen-

zymes have been indicated as muscle and erythrocyte form, respectively, on the basis of the cell-type in which they predominate [9].

The primary structure of the two isoenzymes isolated from different species is known [10]. Basically, each isoenzyme consists of 98 amino acid residues, and can therefore be considered among the smallest enzymes known at present. The comparison of the amino acid sequences of the muscular isoenzymes from different species shows that this form is highly conserved, eliciting only about 10% amino acid substitutions between mammalian and avian species [10]. It has also been found that the NH_2 -terminus, which is always acetylated, is the most variable region of the enzyme. In some cases, the N-terminus contains additional residues, as it is found in the avian species, or is shorter, as in the rat enzyme [11].

The solution structure of the muscular isoenzyme has been determined by ^1H NMR spectroscopy and recently refined [12–15]. The enzyme consists of two interleaved packing units [16] with a quite uncommon folding pattern and chain topology, described only in few proteins such as ferredoxin, the activation domain of procaryo-peptidase B, the RNA binding domain of the small nuclear ribonucleoprotein A [14,15] and a histidine containing phosphocarrier protein [17]. The enzyme three-dimensional structure agrees with the primary structure data, confirming the apparent reduced importance of the N-terminal region, that is not constrained in any persistent secondary structure and appears highly mobile.

In spite of the large amounts of information available on the enzyme structure, little is known about the catalytic mechanism of both isoenzymes. In a study concerning the catalytic mechanism of the muscle enzyme [18], it was shown that a phosphate binding group with a pK_a of about 11.0 is likely to participate to the catalytic cycle together with a group with a pK_a of 7.9 and, possibly, with an -OH group. Recently, it has been shown the participation of Arg-23 in substrate binding at the phosphate level [19]. Previous data indicated that the two C-terminal residues of muscle acylphosphatase cannot be removed without loss of activity [20]. In fact, the Arg-Tyr C-terminal dipeptide is part of a short β -strand [14], and its removal is accompanied by a complete loss of activity, whereas the removal of the C-terminal Tyr results in a remarkable reduction of the enzyme specific activity [20].

It appeared interesting to investigate the possibility of a reduction in length of the enzyme at the N-terminus without loss of biological activity. We therefore prepared a muscle acylphosphatase derivative lacking the first six residues at the N-terminus by oligonucleotide-directed mutagenesis. We also studied two mutants containing the Arg-97 to Gln or Tyr-98

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Abbreviations: Y98Q, tyrosine 98 to glutamine mutant; R97Q, arginine 97 to glutamine mutant; $\Delta 6$, lacking residues 1–6 at the N-terminus; IPTG, isopropyl-thiogalactoside; CED-, cyanoethyldeoxy-; NMR, nuclear magnetic resonance.

to Gln substitutions, respectively, to further elucidate the role of these residues in the enzyme activity.

2. Materials and methods

2.1. Materials

CNBr-activated Sepharose 4B was purchased from Pharmacia; benzoylphosphate was synthesized according to Camici et al. [21]. Specific polyclonal anti-horse muscle acylphosphatase antibodies were obtained and purified according to Berti et al. [22]. [γ - 32 P]ATP and [α - 32 P]ATP (6000 Ci/mmol) were obtained from New England Nuclear. Horseradish peroxidase-linked mouse anti-rabbit Ig was obtained from Bio-Rad. T4 DNA ligase, Klenow fragment of *E. coli* DNA polymerase I, Taq polymerase and restriction enzyme *Nde*I were purchased from Promega. Isopropyl-thiogalattoside (IPTG) was from Promega. USB-Sequenase was used for DNA sequencing. pT7.7 was a gift from Sclavo (Siena, Italy). The plasmid pTAP, containing a chemically synthesized polynucleotide sequence for human muscle acylphosphatase [23], was used for enzyme mutagenesis. Bacterial strain DH5 was used for propagation of the plasmid, and BL21 for deleted proteins expression. Oligonucleotides were synthesized using a Beckman system 200 A DNA synthesizer. Deuterium oxide and d_3 -acetic acid were purchased from Merck.

2.2. Enzyme expression

$\Delta 6$ deleted gene was obtained by polymerase chain reaction, whereas Tyr-98 mutant to Gln (Y98Q) and Arg-97 mutant to Gln (R97Q) were obtained by oligonucleotide-directed mutagenesis according to Mullis et al. [24]. A direct primer starting from the seventh codon was used to obtain the $\Delta 6$ deleted gene (Table 1). This primer, annealing to the synthetic gene coding for human muscle acylphosphatase [23], was designed in order to obtain a correct initiation codon for the translation, after cDNA cloning in the pT7.7 plasmid. The T7 reverse primer annealing to pT7.7 region downstream the recombinant protein coding sequence was used for the amplification. Two reverse primers containing the desired mutation, CGC (Arg) to CAA (Gln) and TAT (Tyr) to CAA (Gln) were used to construct the Arg-97 and Tyr-98 mutants, respectively (Table 1). For these amplifications, the T7 direct primer, annealing to the pT7.7 sequence before the *Nde*I restriction site, was used. All primers were synthesized using the CED-phosphoramidite method.

The deleted gene $\Delta 6$ and the two R97Q and Y98Q mutant genes were

Table 1

Sequences of the oligonucleotides used for the production of the mutated enzymes

Mutant	Oligonucleotide sequence
$\Delta 6$	5' TGAAGTCTGTCGATTACG 3'
R97Q	5' AGCTTTAATAT T GGAATAGAAAAG 3'
Y98Q	5' AAGCTTTAT T GCGGAATAGAAA 3'

The oligonucleotides were synthesized using the CED-phosphoramidite method on a Beckman system 200A DNA synthesizer. The triplets corresponding to the mutated residues (CAA = Gln) are in bold.

Table 2

Main kinetic parameters of wild-type and mutated recombinant acylphosphatases

	Wild-type	R97Q	Y98Q	$\Delta 6$
Specific activity	7000	4700	3600	2500
pH optimum	4.8–5.8	3.7–4.4	5.0–5.9	4.7–5.9
K_m^*	0.51	1.14	0.41	0.55
K_i^*	0.98	1.79	1.17	1.19

The pH optimum was calculated at 25°C, in 0.1 M acetate buffer pH 3.7–6.0 and in 50 mM 3,3-dimethylglutarate buffer pH 2.8–3.7 and 6.0–7.5. The other parameters were determined at 25°C in 0.1 M acetate buffer, pH 5.3 for all mutants, except R97Q which was assayed in the same buffer, pH 3.8. *Using benzoylphosphate as substrate. *For inorganic phosphate as competitive inhibitor.

Fig. 1. Western blot analysis of the wild-type, deleted, and mutated recombinant acylphosphatases. Lane A, molecular weight standards; lane B, wild-type; lane C, $\Delta 6$; lane D, R97Q; lane E, Y98Q.

cloned separately in the *Nde*I single restriction site of the pT7.7 vector, treated with Klenow. The recombinant clones were isolated by hybridisation. All mutants were confirmed by DNA sequencing according to Sanger et al. [25]. *E. coli* strain BL21 was separately transformed with the modified sequences and the expression of the mutated proteins was obtained after induction with IPTG. Western blot analyses were performed according to Tsang et al. [26] using rabbit anti-recombinant human muscle acylphosphatase Ig and horseradish peroxidase-linked mouse anti-rabbit Ig as the second antibody.

2.3. Enzyme purification and protein determination

Recombinant wild-type and mutated acylphosphatases were purified by immunoaffinity chromatography using a column filled with an immunoabsorbent prepared by linking purified specific anti-horse muscle acylphosphatase antibodies, raised in rabbits, to a Sepharose 4B matrix, as previously reported [27]. The enzyme purity was assessed by amino acid analysis, reverse-phase chromatography, and SDS-PAGE. Protein concentration was measured by either amino acid analysis or ultraviolet absorption at 280 nm; an $A_{1\text{cm},280}^{1\%} = 14.20$ for both native and mutated acylphosphatases was used, except for the Y98Q mutant whose concentration was calculated only by amino acid analysis. Amino acid analyses were performed using a Carlo Erba mod. 3A29 amino acid analyzer equipped with a Spectra Physics SP4100 computing integrator, as previously described [28]. Values for serine and threonine were corrected as for degradation by extrapolation to zero hydrolysis time.

2.4. Enzyme activity measurements

Native and mutated acylphosphatase activities were determined using benzoylphosphate as a substrate as previously described [29]. The urea used in the inactivation experiments was previously deionised by treatment with an AG501X8 ion exchange resin (Bio-Rad).

2.5. ^1H NMR analysis

The $\Delta 6$, R97Q and Y98Q samples were prepared by five dilution/concentration cycles in 50 mM d_3 -acetate/ D_2O buffer at pH 3.8 using Centricon-3 microconcentrators (Amicon). pH electrode readings were not corrected for isotope effect. The protein concentration was about 0.5 mM in all samples. One-dimensional ^1H NMR experiments were performed at 600 MHz on a AMX600 Bruker NMR spectrometer, at 30°C. The spectra were acquired using a sweep width of 7246 Hz and 16384 data points. The residual water signal was suppressed by low power irradiation during the relaxation delay (1.0 s). Prior to Fourier transformation, the free induction decays were zero-filled to 32768 data points and multiplied by a resolution-enhancing Gaussian function (with the maximum of the Gaussian set to 0.1 and a line broadening factor of -2 Hz). 1,4-dioxane was used as an internal shift reference at 3.74 ppm.

3. Results

A deleted synthetic gene for acylphosphatase, lacking the first six amino acids from the protein N-terminus (mutant $\Delta 6$) was prepared together with two mutated genes where the Arg-

Fig. 2. Plot of the specific activity of recombinant muscle acylphosphatases vs. urea concentration at pH 5.3 and 25 °C. ○: wild-type; ●: R97Q mutant; ×: Y98Q mutant; △: Δ6 deleted mutant. Inset: same data expressed as % activity, where 100% is the activity of the single species in the absence of urea.

97 and Tyr-98 codons were replaced by those for Gln (mutants R97Q and Y98Q, respectively). All genes were inserted into the *E. coli* expression vector pT7.7 and amplified in *E. coli* strain DH5. *E. coli* strain BL21 was transformed with the recombinant plasmids (pAP Δ6, pAP R97Q, pAP Y98Q) and supplemented with 0.5 mM IPTG to induce recombinant protein expression. The bacterial cells were lysated by sonication, the proteins present in the supernatant separated by 15% SDS-PAGE and subjected to Western blot analysis using polyclonal anti-recombinant muscle acylphosphatase antibodies (Fig. 1). Four bands were present, corresponding to the wild-type acylphosphatase and to the mutated proteins; a negative control was also prepared consisting of the strain BL21 transformed with the pT7.7 without the insert (data not shown). The mutant acylphosphatases present in the lysates were purified as described in section 2. The purified mutant acylphosphatases were characterised as for kinetic properties, stability, and structure. Table 2 reports the main kinetic parameters of the mutant enzymes. All acylphosphatase mutants elicited specific activity values ranging from 35% to 66% with respect to the wild-type enzyme. All mutants and wild-type recombinant acylphosphatases showed apparent K_m values very similar to each other; the R97Q mutant pH-optimum was slightly shifted towards acidity.

The urea denaturation of the mutated and wild-type acylphosphatases was studied at both pH 5.3 and pH 3.8. The activities of all urea-inactivated enzymes were almost completely restored when measured in the absence of urea; this behaviour agrees with that of the non-recombinant muscular isoenzyme [30,31]. Fig. 2 shows the urea dependence of the inactivation of the wild-type and mutated enzymes in 0.1 M acetate buffer at pH 5.3 and 25 °C. It can be seen that the Δ6 mutant behaves quite similarly to the wild-type recombinant enzyme. The urea concentration at which these forms are 50% inactivated (inactivation midpoint) is about 4.2 M. The Y98Q mutant appeared slightly more sensitive to urea, showing an inactivation midpoint at 3.8 M. Instead, at low urea concentrations, the R97Q mutant showed a sharp activation which reached a maximum (about 50%) at 3 M urea, followed by a rapid inactivation leaving 20% active, and completely inactive enzyme at 6 M and 8 M urea, respectively. The specific activity of the R97Q mutant in the presence of 3 M urea was nearly the same as compared to that of the wild-type enzyme under the same conditions (Fig. 2). Similar inactivation experiments were carried out in 50 mM acetate buffer, pH 3.8, both at 25 and 37 °C. The results of such inactivation studies are summarised in Table 3.

¹H NMR spectra were recorded to assess possible changes

Table 3
Urea inactivation experiments of wild-type and mutated acylphosphatases

	pH 5.3, 25°C		pH 3.8, 25°C		pH 3.8, 37°C	
	c_m	Activation	c_m	Activation	c_m	Activation
Wild-type	4.2 M	No	2.6 M	No	2.0 M	No
R97Q	5.2 M	50%, 3 M	3.0 M	25%, 1.5 M	2.5 M	No*
Y98Q	3.8 M	No	2.6 M	No	2.0 M	No
Δ6	4.2 M	No	2.6 M	No	2.0 M	No

The enzymes were pre-incubated for 60 min and subsequently assayed in 50 mM acetate buffer, in the presence of urea concentrations ranging between 0 and 8 M. c_m is the urea concentration required to reduce the enzyme activity to 50% of the value calculated in the absence of urea. Activation, where present, is expressed as % of the enzymatic activity in the absence of urea. The urea concentration at which the activation is maximum is also reported. *Under this condition there is no activation, however, the R97Q mutant maintains the initial activity up to 1.5 M urea.

Fig. 3. Aromatic and aliphatic regions of the 600 MHz ^1H NMR spectra of recombinant acylphosphatases. The samples were in 50 mM d_3 -acetate buffer/ D_2O , pH 3.8 at 30 °C. A: wild-type recombinant enzyme; B: $\Delta 6$ deleted acylphosphatase; C: R97Q mutant; D: Y98Q mutant.

in the fold of the different acylphosphatase forms. Fig. 3 shows the aliphatic and aromatic proton regions of the 600 MHz ^1H NMR spectra of all investigated enzymes. A remarkable chemical shift dispersion is characteristic of a protein with a well-constrained fold, i.e. a native, biologically active protein. A recent study demonstrated that the ^1H NMR spectrum of the wild-type recombinant acylphosphatase is very similar to that of the non-recombinant enzyme [23], confirming a high degree of structural homology between these forms. As can be observed in Fig. 3, the ^1H NMR spectra of the $\Delta 6$ and R97Q mutants show a chemical shift dispersion closely resembling that found in the spectrum of the wild-type recombinant enzyme. Only minor changes in chemical shift of both aromatic and aliphatic resonances are evident from these one-dimensional spectra. The Y98Q mutant ^1H NMR spectrum, though maintaining the overall chemical shift dispersion, shows some resonances characteristic of the random-coil conformation [31,32], in particular the methyl proton and the tyrosine 3,5 ring protons peaks at about 1.0 and 6.8 ppm, respectively, are present. Moreover, in the same spectrum, the intensity of the aliphatic resonances below 0.6 ppm, characteristic of the native fold, are considerably reduced.

4. Discussion

The $\Delta 6$, R97Q and Y98Q mutated acylphosphatases studied in this paper elicit a reduced specific activity with respect to the wild-type recombinant enzyme, whereas the apparent K_m value is nearly identical. This data is confirmed by the ^1H NMR spectra of all enzyme forms. As far as the $\Delta 6$ deletion mutant is concerned, it has previously been reported that the 1–6 segment is highly mobile and apparently not involved in the formation of any secondary structure element [16]. Nevertheless, the

sharp reduction of the specific activity of this mutant, indicates that the removal of the 1–6 segment somewhat affects the active site structure. The ^1H NMR spectrum of this mutant shows the absence of any major conformational change, indicating that the loss of catalytic activity is probably due to minor local structural modifications at the active site that do not affect the substrate binding.

The R97Q and Y98Q mutants possess specific activities which are about 66% and 50% that of the wild type recombinant enzyme, respectively. The R97Q ^1H NMR spectrum is similar to that of the wild-type recombinant enzyme, indicating that the overall folds of the two enzymes are basically identical. Instead, the Y98Q ^1H NMR spectrum shows the presence of intense resonances, characteristic of the denatured acylphosphatase [19,31], that are not present in the wild-type enzyme spectrum. A partial loss of tertiary structure of this mutant could explain the appearance of these peaks and the concomitant reduction in intensity of the resonances below 0.6 ppm. In the light of these structural findings, the reduced specific activities of both mutants could be ascribed to different effects of the residue replacements. Tyr-98 appears a critical residue for the maintenance of the fully native-like structure whereas the R97Q reduced specific activity might be due to a local effect on the active site stability. Nevertheless, these residues are not likely to affect substrate binding site structure, in view of the identity of the apparent K_m value. In a previous paper, it was shown that the enzyme derivative lacking the Arg-97–Tyr-98 C-terminal dipeptide was completely inactive though apparently eliciting a global native-like fold [20]. These results led to hypothesize that Arg-97 could be involved in the enzyme catalytic mechanism. However, our data demonstrates that the Arg-97 mutation is not accompanied by a complete loss of enzymatic activity. This apparent contradiction can be explained assuming that the removal of the C-terminal dipeptide destabilises the active site structure. In fact, this dipeptide is part of the fifth β -strand, forming an hydrogen bonding network to other secondary structure elements [14]. The simple mutation of the Arg-97 residue does not seem severe enough to determine the same effects of the C-terminal dipeptide deletion.

The results of the urea inactivation experiments show that the $\Delta 6$ deletion mutant and the Y98Q mutant behave similarly to the wild-type recombinant enzyme both at pH 5.3 and pH 3.8. However, all three forms are less stable when compared with the native horse muscle enzyme [31]. This difference could be explained by considering that the recombinant and native acylphosphatases were from different species (human and horse, respectively) and, more important, that the recombinant enzymes are not acetylated at the N-terminus. The R97Q mutant shows a peculiar behaviour appearing activated up to 1.5 M and 3 M urea, at pH 3.8 and 5.3, respectively. The activation is such that the specific activity of this mutant becomes very similar to that of the wild-type recombinant enzyme, when measured under the same conditions. Thus, in the presence of urea, the enzyme activity appears no longer affected by the arginine substitution. This finding could be explained by the existence of a persistent interaction between urea and the Gln-97 side-chain; the urea–Gln-97 complex would be able to mimic the interactions of Arg-97 to other parts of the molecule stabilising the correct enzyme conformation at the active site, and favouring enzyme catalysis. A recent refinement of the acyl-

phosphatase solution structure revealed that the Arg-97 side-chain interacts with other residues (A. Pastore, personal communication), in particular with Arg-23, whose side-chain participates to the substrate binding site [19]. The structural and physico-chemical features of such an Arg–Arg interaction in proteins have recently been investigated [33]. The absence of urea activation in the Y98Q mutant, indicates that this effect is specific for Arg-97.

The reported results suggest that Arg-97 and Tyr-98 are not directly involved in the acyl-phosphatase catalytic mechanism or substrate binding. Instead, these residues appear involved in the stabilisation of the active site (Arg-97) and of the protein global conformation (Tyr-98). The contribution to this stabilisation by each residue is limited, as demonstrated by the specific activity value of the two mutants and of the enzyme lacking Tyr-98 [20]. However, the sum of these contributions appears essential for enzyme activity, as suggested by the complete enzyme inactivation following the removal of the C-terminal dipeptide [20]. Our data indicate that acylphosphatase cannot be shortened either at the N- and C-terminus without partial or complete loss of activity, though the C-terminal region appears more important for the maintenance of the correct fold of the enzyme.

Acknowledgements: This work was supported by grants from MURST (60%), from CNR (Target Project on Bio-technology and Bioinstrumentation), and from the Italian Cancer Research Association (AIRC). The authors thank Mr. Massimo Lucci and Mr. Enrico Morelli for technical assistance in performing the NMR spectra and Dr. Luigia Pazzagli for amino acid analyses. The NMR spectra were recorded at the Laboratorio di Risonanze Magnetiche of the University of Florence.

References

- [1] Ramponi, G. (1975) *Methods Enzymol.* 42, 409–435.
- [2] Berti, A., Stefani, M., Liguri, G., Camici, G., Manao, G. and Ramponi, G. (1977) *Ital. J. Biochem.* 26, 377–378.
- [3] Hokin, I.E., Sastry, P.S., Galsworthy, P.R. and Yoda, A. (1965) *Proc. Natl. Acad. Sci. USA* 54, 177–184.
- [4] Stefani, M., Liguri, G., Berti, A., Nassi, P. and Ramponi, G. (1981) *Arch. Biochem. Biophys.* 208, 37–41.
- [5] Nassi, P., Nediani, C., Liguri, G., Taddei, N., Ruggiero, M. and Ramponi, G. (1990) *Biochem. Biophys. Res. Commun.* 168, 651–658.
- [6] Nassi, P., Nediani, C., Liguri, G., Taddei, N. and Ramponi, G. (1992) *J. Biol. Chem.* 266, 10867–10871.
- [7] Nassi, P., Marchetti, E., Nediani, C., Liguri, G. and Ramponi, G. (1993) *Biochim. Biophys. Acta* 1147, 19–26.
- [8] Liguri, G., Camici, G., Manao, G., Cappugi, G., Nassi, P., Modesti, A. and Ramponi, G. (1986) *Biochemistry* 25, 8089–8094.
- [9] Degl'Innocenti, D., Berti, A., Stefani, M. and Ramponi, G. (1990) *Biotech. Appl. Biochem.* 12, 450–459.
- [10] Pazzagli, L., Cappugi, G., Camici, G., Manao, G. and Ramponi, G. (1993) *J. Prot. Chem.* 12, 593–601.
- [11] Berti, A., Tremori, E., Pazzagli, L., Degl'Innocenti, D., Camici, G., Cappugi, G., Manao, G. and Ramponi, G. (1991) *J. Prot. Chem.* 10, 91–102.
- [12] Saudek, V., Williams, R.J.P. and Ramponi, G. (1988) *J. Mol. Biol.* 199, 233–237.
- [13] Saudek, V., Atkinson, R.A., Williams, R.J.P. and Ramponi, G. (1989) *J. Mol. Biol.* 205, 229–239.
- [14] Saudek, V., Wormald, M.R., Williams, R.J.P., Boyd, J., Stefani, M. and Ramponi, G. (1989) *J. Mol. Biol.* 207, 405–415.
- [15] Pastore, A., Saudek, V., Ramponi, G. and Williams, R.J.P. (1992) *J. Mol. Biol.* 224, 427–440.
- [16] Saudek, V., Williams, R.J.P. and Ramponi, G. (1989) *FEBS Lett.* 242, 225–232.
- [17] Swindells, M.B., Orengo, C.A., Jones, D.T., Pearl, L.H. and Thornton, J.M. (1993) *Nature* 362, 299.
- [18] Satchell, D.P.N., Spencer, N. and White, G.F. (1972) *Biochim. Biophys. Acta* 268, 233–248.
- [19] Taddei, N., Stefani, M., Vecchi, M., Modesti, A., Raugei, G., Bucciantini, M., Magherini, F. and Ramponi, G. (1994) *Biochim. Biophys. Acta* 1208, 75–80.
- [20] Stefani, M., Cappugi, G., Pazzagli, L., Camici, G., Manao, G., Taddei, N., Buck, M. and Ramponi, G. (1991) *Int. J. Peptide Protein Res.* 38, 278–284.
- [21] Camici, G., Manao, G., Cappugi, G. and Ramponi, G. (1976) *Experientia* 32, 535.
- [22] Berti, A., Liguri, G., Stefani, M., Nassi, P. and Ramponi, G. (1982) *Physiol. Chem. Phys.* 14, 307–311.
- [23] Modesti, A., Raugei, G., Taddei, N., Marzocchini, R., Vecchi, M., Camici, G., Manao, G. and Ramponi, G. (1993) *Biochim. Biophys. Acta* 1216, 369–374.
- [24] Mullis, F., Faloon, F., Scharf, S., Saiki, R., Horn, G., and Elrich, H. (1986) *Cold Spring Harbor Symp. Quant. Biol.* 51, 263–273.
- [25] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA* 74, 5436.
- [26] Tsang, V.C.W., Peralta, J.M. and Simons, A.R. (1983) *Methods Enzymol.* 92, 377–391.
- [27] Manao, G., Camici, G., Stefani, M., Berti, A., Cappugi, G., Liguri, G., Nassi, P. and Ramponi, G. (1983) *Arch. Biochem. Biophys.* 226, 414–424.
- [28] Manao, G., Camici, G., Cappugi, G., Stefani, M., Liguri, G., Berti, A. and Ramponi, G. (1985) *Arch. Biochem. Biophys.* 241, 418–424.
- [29] Ramponi, G., Treves, C. and Gueritore, A. (1966) *Experientia* 22, 1019–1023.
- [30] Berti, A., Stefani, M., Camici, G., Manao, G. and Ramponi, G. (1978) *Physiol. Chem. Phys.* 13, 267–273.
- [31] Taddei, N., Buck, M., Broadhurst, R.W., Stefani, M., Ramponi, G. and Dobson, C.M. (1994) *Eur. J. Biochem.* 225, 811–817.
- [32] Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York.
- [33] Magalães, A., Maigret, B., Hoflack, J., Gomes, J.N.F. and Scheraga, H.A. (1994) *J. Prot. Chem.* 13, 195–215.