FEBS Letters 384 (1996) 172–176 FEBS 16903

C-terminal region contributes to muscle acylphosphatase three-dimensional structure stabilisation

Niccolò Taddei, Francesca Magherini, Fabrizio Chiti, Monica Bucciantini, Giovanni Raugei, Massimo Stefani*, Giampietro Ramponi

Department of Biochemical Sciences, University of Florence, V.le Morgagni 50, 50134 Florence, Italy

Received 7 February 1996; revised version received 6 March 1996

Abstract Ser-Ala and Ser-Ala-Ser-Ala C-terminus elongated (Δ+2 and Δ+4, respectively) and two C-terminus deleted (Δ−2 and Δ−3) muscle acylphosphatase mutants were investigated to assess the catalytic and structural roles of the C-terminal region. The kinetic analysis of these mutants shows that the removal of two or three C-terminal residues reduces the catalytic activity to 7% and 4% of the value measured for the wild-type enzyme, respectively; instead, the elongation of the C-terminus does not significantly change the enzyme behaviour. ^{1}H Nuclear magnetic resonance spectroscopy indicates that all mutants display a native-like fold though they appear less stable, particularly Δ−2 and Δ−3 mutants, as compared to the wild-type enzyme. Such destabilisation of the C-terminal modified mutants is further confirmed by urea inactivation experiments. The results here presented account for an involvement of the C-terminal region in the stabilisation of the three-dimensional structure of acylphosphatase, particularly at the active-site level. Moreover, a participation of the C-terminal carboxyl group to the catalytic mechanism can be excluded.

Key words: Acylphosphatase, deletion mutant; Acylphosphatase, insertion mutant; Acylphosphatase, stability; Acylphosphatase, ^{1}H NMR spectrum

1. Introduction

Acylphosphatase (E.C. 3.6.1.7) is a very small protein (M_r 11 365, horse muscle enzyme) widely distributed in vertebrate tissues in the form of two isozymes named muscle and erythrocyte (common) type from the tissue/cell in which they are most abundant or from which they were first purified [1]. The two isozymes share a high sequence homology ranging from over 55% in mammals to about 62% and 70% in birds and fish, respectively ([2] and unpublished results). The two isozymes are probably derived from a common ancestor gene by duplication and subsequent evolution. Acylphosphatase isozymes catalyse the hydrolysis of carboxylphosphate bonds present in physiologically important compounds such as 1,3-*bis*phosphoglycerate, carbamoylphosphate, succinylphosphate, and the β-aspartylphosphate formed during the activity of membrane ion pumps [1]. It has been suggested that the enzyme interferes with the glycolytic flux and enhances the rate of alcohol fermentation [3]. Both acylphosphatase isozymes exert a significant effect on membrane ion transport systems [4,5]. Moreover, muscular acylphosphatase expression in cultured cells appears as an early differentiation event in the myoblast/myotube system [6].

The three-dimensional solution structure of the muscular isozyme has been determined by ^{1}H NMR spectroscopy [7]. The enzyme is a closely packed α/β protein composed of five β-strands forming an antiparallel, twisted β-sheet facing two antiparallel α-helices. The secondary structure elements do not follow each other in sequence and can be grouped into two βαβ units interleaved into each other. This fold has been found in proteins such as the bacterial histidine-containing phosphocarrier protein (HPr), the bacterial mercuric ion binding protein (MerP), the RNA binding domain of the small nuclear ribonucleoprotein A, and a few others [8–11].

Limited information is currently available on both the enzyme kinetic mechanism and the residues involved in catalysis [12]. Recently, two catalytic residues have been identified by oligonucleotide-directed mutagenesis ([13] and manuscript submitted for publication). In fact, mutants at Arg-23 or Asn-41 elicited a dramatically reduced (over 2000-fold) catalytic efficiency though maintaining the overall native-like fold. Arg-23 mutants were completely unable to bind the substrate/competitive inhibitor [13], whereas Asn-41 mutants fully maintained this ability (manuscript submitted for publication).

Previous ^{1}H NMR findings indicated the possible location of the enzyme active site in the proximity of the C-terminus region [14], which is part of the fifth β-strand of the β-sheet. Later, a nearly complete loss of activity was shown in the muscle isozyme lacking the two C-terminal residues (Arg-97 and Tyr-98), previously removed by carboxypeptidase treatment [15]. More recently, the individual importance of Arg97 and Tyr98 for enzyme activity and stability has been studied by the same oligonucleotide-directed mutagenesis approach [16], indicating that Arg-97 and Tyr-98 do not directly participate in the catalytic mechanism. Nevertheless, these studies did not assess a possible involvement of the carboxy-terminal group in catalysis.

Two deletion (Δ−2 and Δ−3) and two insertion (Δ+2 and Δ+4) enzyme mutants were prepared by oligonucleotide-directed mutagenesis. These mutants were studied to definitely understand the role of both the C-terminal region and the carboxy-terminal group in the muscle-type acylphosphatase active site function and stabilisation.

*Corresponding author. Fax: (39) (55) 4222725.
E-mail: dpt_bioch@cesit1.unifi.it

Abbreviations: Δ+2, acylphosphatase mutant containing a Ser-Ala extension at the C-terminus; Δ+4, acylphosphatase mutant containing a Ser-Ala-Ser-Ala extension at the C-terminus; Δ−2, acylphosphatase mutant lacking the C-terminal dipeptide; Δ−3, acylphosphatase mutant lacking the C-terminal tripeptide; IPTG, isopropyl thiogalactoside; GST, glutathione *S*-transferase; NMR, nuclear magnetic resonance.

S0014-5793/96/$12.00 © 1996 Federation of European Biochemical Societies. All rights reserved.
SSDI S0014-5793(96)00292-X

2. Materials and methods

2.1. Materials

Benzoylphosphate was synthesised as previously described [17] and dissolved immediately prior to enzyme assays. Specific polyclonal anti-recombinant human muscle acylphosphatase antibodies used in the Western blot experiments were obtained according to Berti et al. [18]. Glutathione, human thrombin, and glutathione-agarose affinity gel were from Sigma. D_2O and d_4-acetic acid were from Merck (Darmstadt). Klenow fragment of *E. coli* DNA polymerase I, T4 DNA ligase, Taq polymerase, and restriction enzymes *Bam*HI and *Eco*RI were purchased from Promega; sequenase was from USB; pGEX-2T vector was from Pharmacia; isopropyl thiogalactoside (IPTG) was from Boehringer (Mannheim). Horseradish peroxidase-linked mouse anti-rabbit Ig was from Bio-Rad. [α-^{32}P]ATP (800 Ci/mmol) was from New England Nuclear. The pGEX-AP plasmid was obtained as previously described from the pGEX-2T vector harbouring a chemically synthesised gene coding for human muscle acylphosphatase [19]. The DH5α and TB1 bacterial strains were used for plasmid propagation and recombinant fusion protein expression, respectively. Chemically synthesised oligonucleotides containing the desired insertions and deletions were obtained from Pharmacia. All other reagents were analytical grade or the best commercially available.

2.2. Oligonucleotide-directed mutagenesis

Δ−2 and Δ−3 deleted genes together with Δ+2 and Δ+4 genes were obtained by polymerase chain reaction according to Mullis et al. [20]. A direct primer harbouring the restriction site for *Bam*HI and annealing to the synthetic gene coding for human muscle acylphosphatase was designed in order to obtain a correct insertion in the plasmid pGEX-2T. Two reverse primers harbouring a stop codon instead of the codon for the 97th and 96th amino acid (5′-TCAAATAGA-AAAGTTGCTGTA-3′, and 5′-AATAGCGTTAAGAAAAGTTGC-3′) were used in order to obtain the Δ−2 and Δ−3 deleted genes, respectively. Two reverse primers containing two and four extra codons at the acylphosphatase gene sequence corresponding to the enzyme C-terminus (5′-GCGTTAGGCGCTATAGCGAATAGAAAG-3′ and 5′-GCGTTAGGCGCTGGCGCTATAGCGAATAGAAAA-GTTGCTG-3′) were used in order to obtain the Δ+2 and Δ+4 genes, respectively.

2.3. Fusion protein expression and enzyme purification

The mutated sequences were treated with Klenow enzyme and then digested with *Bam*HI restriction enzyme. The obtained fragments were separately ligated into the pGEX-2T plasmid previously digested with *Eco*RI, treated with Klenow and then digested with *Bam*HI. The fragments were then inserted downstream and in frame with the glutathione *S*-transferase coding sequence. The recombinant clones were isolated by colony hybridisation. All mutations were confirmed by DNA sequencing according to Sanger et al. [21]. Mutated protein expression was obtained in the *E. coli* strain TB1 after induction with IPTG. The cultures were grown at 37°C under shaking up to stationary phase, and the presence of the mutated proteins in cell lysates was checked by SDS-PAGE. The GST-acylphosphatase fusion proteins were purified from cell lysates as previously reported [19] with minor modifications in order to obtain the enzyme in the reduced (−SH) form. The purified enzymes were controlled by Western blot analysis. Thrombin cleavage was performed by incubating each fusion protein with 1:1000 (w/w) human thrombin in 50 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl and 2.5 mM $CaCl_2$; incubation was performed at room temperature for 1 h. Each cleaved enzyme was separated from GST and uncleaved fusion protein by gel filtration as previously reported [19]. Fractions containing the cleaved enzyme were pooled, concentrated and analysed for purity by SDS-PAGE.

2.4. Protein determination and amino acid analysis

Protein concentration was determined by UV absorption using an $A^{1\%}_{1cm,280} = 14.2$; for the C-terminus deleted enzymes, lacking Tyr98, an $A^{1\%}_{1cm,280} = 13.8$ was calculated. Amino acid analyses were performed by a Carlo Erba model 3A29 amino acid analyser equipped with an Hewlett Packard HP3395 computing integrator. Values for threonine and serine were corrected for residue degradation during sample hydrolysis.

2.5. Acylphosphatase activity measurements

Acylphosphatase activity was measured by a continuous optical test at 283 nm and 25°C using benzoylphosphate as a substrate, as previously described [22]. The assay test contained 5 mM benzoylphosphate in 0.1 M acetate buffer, pH 5.3, unless otherwise stated. Deletion mutant activity was measured in the presence of an enzyme concentration (0.010–0.015 mg/ml) 10–100-fold higher than that used in the standard assay.

2.6. Urea inactivation experiments

Urea inactivation experiments were carried out at 37°C in 50 mM acetate buffer, pH 3.8. The urea stock solution was deionised just before the experiments by incubation with an AG501X8 ion exchange resin (Bio-Rad) for 30 min. The samples were preincubated for 1 h in the presence of differing urea concentrations (ranging from 0 to 8 M). Enzyme activity was measured in assay mixtures containing the corresponding urea concentrations.

2.7. ^1H NMR spectroscopy

The ^{1}H NMR experiments were carried out at 600 MHz using a Bruker AMX600 spectrometer. Protein concentration in samples was made 1–2 mg/ml by dissolving the lyophilised enzymes in 50 mM d_3-acetate buffer/D_2O, pH 3.8. The samples were immediately used for recording one-dimensional ^{1}H NMR spectra using the acquisition parameters previously reported [16]. Protein thermal unfolding was followed by recording spectra at different temperatures ranging from 30 to 70°C. 512 transients were acquired for all samples. 1,4-Dioxane was used as an internal chemical shift reference at 3.74 ppm.

3. Results and discussion

Four synthetic genes coding for human muscle acylphosphatase have been synthesised. Δ−2 and Δ−3 deletion genes corresponded to the wild-type enzyme lacking two and three C-terminal residues, respectively, whereas Δ+2 and Δ+4 inser-

Table 1
Main kinetic and stability parameters of mutated and wild-type acylphosphatase

	pH optimum	Specific activity (IU/mg)	K_m (mM)[a]	K_i (mM)[b]	t_m (°C)	c_m (M)
Wild-type	4.8–5.8	6500	0.36	0.75	53.0	2.65
Δ+2	4.8–5.8	5800	0.37	0.77	52.0	2.25
Δ+4	4.6–5.6	5100	0.39	0.88	50.5	1.90
Δ−2	3.8–4.8	480	0.82	2.85	48.0	1.65
Δ−3	4.0–5.0	280	1.40	4.70	45.5	1.50

IU is defined as the enzyme activity which catalyses the hydrolysis of 1 μmol/min of benzoylphosphate at 25°C and pH 5.3. 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 6.0–7.5. The other kinetic parameters were determined at 25°C in 0.1 M acetate buffer, pH 5.3 (wild-type and insertion mutants) or 4.3 (deletion mutants). c_m is the urea concentration required to reduce the enzyme activity to 50% of the value in the absence of urea at 37°C in 50 mM acetate buffer, pH 3.8. t_m is the temperature needed to denature 50% of the molecules; it was calculated by ^{1}H NMR spectroscopy in 50 mM acetate buffer, pH 3.8, as previously described [26].

[a]Using benzoylphosphate as substrate.

[b]For inorganic phosphate as competitive inhibitor.

tion genes corresponded to that coding for the wild-type enzyme extended by two and four residues at the C-terminus, respectively. The C-terminal extra residues were Ser-Ala (Δ+2) and Ser-Ala-Ser-Ala (Δ+4). The four mutated genes, together with that coding for the wild-type enzyme, were cloned and expressed using the four primers described in section 2. The wild-type and mutant enzymes were purified from cell lysates as described in section 2. The purification yields of the wild-type, Δ+2 and Δ+4, were over 5 mg of protein/liter of cell culture. In the case of the Δ−2 and Δ−3 mutants, the yields of purification were significantly lower (less than 1 mg/l), due to enzyme precipitation into cell and during the purification steps. Fig. 1 shows the SDS-PAGE of the four purified enzyme mutants together with the wild-type one. Table 1 reports the main kinetic data of the purified acylphosphatases. It can be seen that the deletion mutants show markedly reduced specific activities ranging from 7% (Δ−2) to about 4% (Δ−3) as compared to the wild-type enzyme, whereas the insertion mutants display a catalytic activity very similar to that of the wild-type acylphosphatase. The decrease of catalytic activity observed in the deletion mutants is accompanied by a parallel reduction in substrate binding capability. In fact, the apparent K_m and the K_i (for inorganic phosphate, an enzyme competitive inhibitor) values of deletion mutants increase progressively following the C-terminus reduction in length; instead, the substrate affinity elicited by the insertion mutants appears substantially unchanged. This finding suggests that the amino acid residues (particularly Arg-23) involved in substrate binding maintain their correct position in the insertion mutants. In contrast, in the deletion mutants, the active site is likely to suffer a structural perturbation impairing substrate binding. The low residual activity (about 7%) of the Δ−2 mutant partially contrasts with previously reported data indicating that the Tyr-98Arg-97 truncated acylphosphatase derivative was completely inactive [15]. In principle, the fully inactive C-terminus truncated enzyme can be considered identical to the slightly active Δ−2 mutant. However, it should be noted that the truncated derivative was obtained from native acylphosphatase by carboxypeptidase A digestion at 30°C and pH 7.0 followed by HPLC purification of the so obtained *des*-Tyr-98 derivative; the latter was then subjected to carboxypeptidase B digestion at 30°C and pH 7.5. Under these conditions, a partial unfolding of the

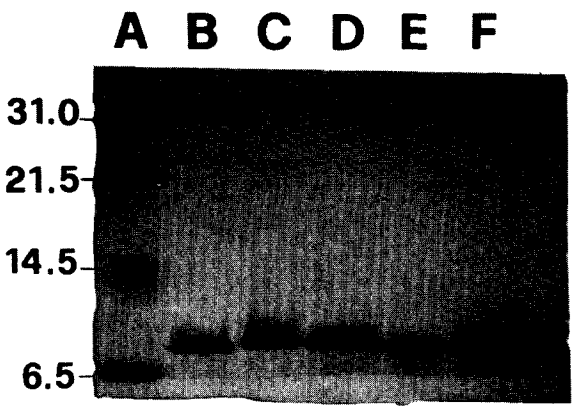

Fig. 1. SDS-PAGE of the purified recombinant acylphosphatases. Lane A: M_r markers; lane B: wild-type enzyme; lane C: Δ+4 mutant; lane D: Δ+2 mutant; lane E: Δ−3 mutant; lane F: Δ−2 mutant.

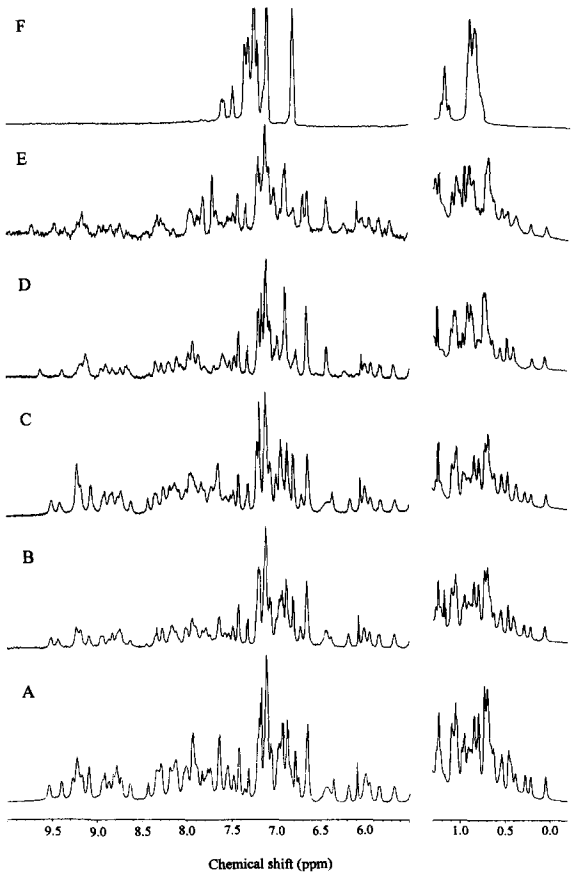

Fig. 2. Backbone amide, aromatic and aliphatic proton regions of the 600 MHz spectra of the wild-type (A), Δ + 4 (B), Δ + 2 (C), Δ−2 (D), Δ−3 (E), acylphosphatases. The spectra were recorded in 50 mM d_3-acetate/D_2O buffer, pH 3.8, at 37°C. (F) Thermally denatured (70°C) wild-type enzyme in the same buffer.

truncated enzyme accompanied by loss of activity is likely to have occurred, explaining the complete enzyme inactivation. This possibility is supported by the reduced stability of the deletion mutants reported in the present study. In another paper, it was shown that acylphosphatase Arg-97 to Gln and Tyr-98 to Gln mutants maintained over 50% and 65% of the wild-type enzyme specific activity, respectively [16]. The sharp loss of catalytic efficiency of both deletion mutants described here cannot be due to an involvement of the C-terminal residues in the catalytic mechanism. Furthermore, the data relative to the insertion mutants make a direct participation of the terminal carboxylate group in the catalytic mechanism unlikely. In fact, in such mutants the elongation of the C-terminus, causing a displacement of the carboxylate group, was not followed by a significant alteration of the kinetic properties.

A ^1H NMR spectroscopy investigation was carried out in order to obtain information about the global fold of the mutants as compared to that of the wild-type. Fig. 2 reports the 600 MHz ^1H NMR spectra of the four enzyme mutants together with those of the wild-type enzyme both in the native and in the thermally denatured (at 70°C) forms. It is well known that the dispersion of proton resonances arises as a consequence of persistent contacts characteristic of a stable tertiary structure [23]. Such a feature is shown by the spectra recorded at 30°C (Fig. 2A–E), confirming the presence of a

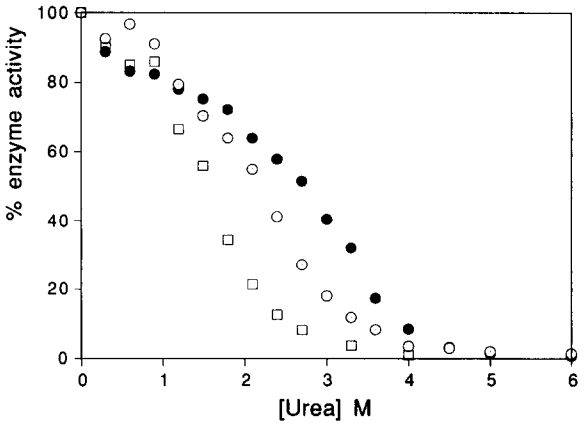

Fig. 3. Plot of the % enzymatic activity of the recombinant acylphosphatases vs. urea concentration. 100% is defined as the activity measured in the absence of urea. ●: wild-type enzyme; ⊙: Δ+2 mutant; □: Δ−3 mutant.

native-like fold in all mutants. This finding allows us to exclude that the relevant loss of activity of the deletion mutants is due to a major unfolding. However, the intensity of the resonances in the backbone amide proton region (approximately above 7.3 ppm) of the deletion mutants spectra is significantly reduced as compared to that of the wild-type enzyme. In this region, the resonances derive from buried protons protected against the exchange with the solvent deuterions. The extent of protection, depending on structural fluctuations within the molecule, reflects the presence of a well constrained fold [24,25]. Our ^{1}H NMR data at 30°C (Fig. 2A–E) suggest that both deletion mutants undergo larger structural fluctuation than the wild-type enzyme. This conclusion is further supported by the behaviour of the different proteins upon heating. The ^{1}H NMR spectra of both mutated and wild-type enzyme were recorded at different temperatures, in the 30–70°C range, using the same acquisition parameters and taking into account the time elapsed after sample resuspension (data not shown). Backbone amide proton resonances completely disappeared in the deletion mutant and wild-type enzyme spectra recorded at 40 and 46°C, respectively, indicating a higher susceptibility of the two mutants to deuterion/proton exchange. This finding is indirect evidence of a lower stability of both deletion mutants as compared to the wild-type enzyme. Furthermore, the analysis of the ^{1}H NMR spectra recorded at different temperatures enabled us to calculate the t_m values (temperature at which 50% of the enzyme molecules are unfolded) for all mutants, following a previously described procedure [26]. Table 1 shows that the insertion mutants are slightly more sensitive to temperature as compared to the wild-type enzyme. The t_m values relative to the deletion mutants indicate that the removal of two or three residues from the C-terminus is responsible for a significant reduction of the enzyme stability.

The stability of all enzymes was further studied using the enzymatic activity as a structural probe. Fig. 3 shows part of the results of inactivation experiments performed at 37°C under increasing urea concentrations. The c_m values (Table 1), defined as the urea concentrations needed to reduce the enzymatic activity to 50% of the value calculated in the absence of urea, suggest that all mutants, particularly the deletion ones, are more sensitive to urea as compared to the wild-type enzyme.

The data reported here, taken together with previous results [15,16], are strongly indicative of an involvement of the C-terminal region in acylphosphatase three-dimensional structure stabilisation. In fact, the deletion and, to a lesser extent, the elongation of the C-terminal region reduce the enzyme stability, as indicated by ^{1}H NMR spectra and urea inactivation experiments. Such destabilisation would occur through the modification of the fifth β-strand which is part of the antiparallel β-sheet. The removal of the last two and three C-terminal amino acid residues is also responsible for both a remarkable loss of activity and a weaker substrate binding capability which are likely to arise from a direct destabilisation of the active site structure, confirming previous ^{1}H NMR data [7] showing the spatial proximity between the two C-terminal residues and catalytically relevant residues, such as Arg-23 and Asn-41. Moreover, the unchanged kinetic properties of the insertion mutants indicate that the terminal carboxylate group does not participate in catalysis.

Acknowledgements: This study has been supported by grants from the Italian MURST (fondi 40%), CNR (Target Project Structural Biology), Telethon (Grant No. 686) and Associazione Italiana per la Ricerca sul Cancro (AIRC). M.B. is recipient of a fellowship from AIRC. ^{1}H NMR spectroscopy experiments were carried out at the Laboratorio di Risonanze Magnetiche of the Università di Firenze. The authors gratefully thank Mr. M. Lucci and Mr. E. Morelli for technical advice.

References

[1] Stefani, M. and Ramponi, G. (1995) Life Chem. Rep. 12, 271–301.
[2] Pazzagli, L., Cappugi, G., Camici, G., Manao, G. and Ramponi, G. (1993) J. Protein Chem. 12, 593–601.
[3] Ramponi, G., Liguri, G., Nediani, C., Stefani, M., Taddei, N. and Nassi, P. (1989) Biotechnol. Appl. Biochem. 10, 408–413.
[4] Nassi, P., Nediani, C., Liguri, G., Taddei, N. and Ramponi, G. (1991) J. Biol. Chem. 266, 10867–10871.
[5] Nediani, C., Fiorillo, C., Marchetti, E., Bandinelli, R., Degl'Innocenti, D. and Ramponi, G. (1995) Biochemistry 34, 6668–6674.
[6] Berti, A., Degl'Innocenti, D., Stefani, M. and Ramponi, G. (1992) Arch. Biochem. Biophys. 294, 261–264.
[7] Pastore, A., Saudek, V., Ramponi, G. and Williams, R.J.P. (1992) J. Mol. Biol. 224, 427–440.
[8] Nagai, K., Oubridge, C., Jesenn, T.H., Li, J. and Evans, P.R. (1990) Nature 348, 515–520.
[9] Hegde, R.S., Grossman, S.R., Laimins, L.A. and Sigler, P.B. (1992) Nature 359, 505–512.
[10] Eriksson, P.O. and Sahlman, L. (1993) J. Biomol. NMR 3, 613–626.
[11] Van Nuland, N.A.J., Hangyi, I.W., Van Schaik, R.C., Berendsen, H.J., Van Gunsteren, W.F., Scheek, R.M. and Robillard, G.T. (1994) J. Mol. Biol. 544–559.
[12] Satchell, D.P.N., Spencer, N. and White, G.F. (1972) Biochim. Biophys. Acta 268, 233–248.
[13] Taddei, N., Stefani, M., Vecchi, M., Modesti, A., Raugei, G., Bucciantini, M., Magherini, F. and Ramponi, G. (1994) Biochim. Biophys. Acta 1208, 75–80.
[14] Saudek, V., Williams, R.J.P. and Ramponi, G. (1989) FEBS Lett. 242, 225–232.
[15] 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.
[16] Taddei, N., Modesti, A., Bucciantini, M., Stefani, M., Magherini, F., Vecchi, M., Raugei, G. and Ramponi, G. (1995) FEBS Lett. 362, 175–179.
[17] Camici, G., Manao, G., Cappugi, G. and Ramponi, G. (1976) Experientia 32, 535.

[18] Berti, A., Liguri, G., Stefani, M., Nassi, P. and Ramponi, G. (1982) Physiol. Chem. Phys. 14, 307–311.
[19] Modesti, A., Taddei, N., Bucciantini, M., Stefani, M., Colombini, B., Raugei, G. and Ramponi, G. (1995) Protein Expres. Purif. 6, 799–805.
[20] Mullis, F., Faloona, F., Scharf, S., Saiki, R., Horn, G. and Elrich, H. (1986) Cold Spring Harbor Symp. Quant. Biol. 51, 263–273.
[21] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5436.
[22] Ramponi, G., Treves, C. and Guerritore, A. (1966) Experientia 22, 1019–1023.
[23] Wüthrich, K. (1986) NMR of Proteins and Nucleic Acids, John Wiley and Son, New York.
[24] Englander, S.W. and Kallenbach, N.R. (1984) Q. Rev. Biophys. 16, 521–655.
[25] Creighton, T.E. (1993) Proteins Structures and Molecular Properties, W.H. Freeman and Co., New York.
[26] Taddei, N., Buck, M., Broadhurst, R.W., Stefani, M., Ramponi, G. and Dobson, C.M. (1994) Eur. J. Biochem. 225, 811–817.