An attempt to transform class characteristics within the alcohol dehydrogenase family

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Abstract Human class I alcohol dehydrogenase was mutated at positions 57 and 115, exchanging for Asp and Arg respectively, in an attempt to introduce glutathione-dependent formaldehyde dehydrogenase characteristics. In addition, class III alcohol dehydrogenase, identical to glutathione-dependent formaldehyde dehydrogenase, was mutated at position 115, introducing Ser or Lys. The attempted class transformation was partly successful considering a higher affinity for 12-hydroxydodecanoate and a lower affinity for ethanol that was monitored for the class I mutant. However, the class I mutant displayed neither glutathione-dependent formaldehyde dehydrogenase activity nor fatty acid activation of alcohol oxidation. Interestingly, both class III mutants showed reduced activities for *S*-hydroxymethylglutathione and 12-hydroxydodecanoate through increased K_m values. Overall results show that it is not possible, by single point mutations, to completely transform enzyme characteristics between these two classes of alcohol dehydrogenase.

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Key words: Alcohol dehydrogenase; Recombinant protein; Mutagenesis; Enzyme kinetics

1. Introduction

All cells are under a continuous pressure from different toxic substances. For protection, different detoxifying enzyme systems have evolved such as the medium chain zinc-containing alcohol dehydrogenases (ADH). They are found in almost all organisms and comprise a large family of enzymes that are divided into several classes [1]. Two of these classes, class I and III, have been investigated in a vast number of vertebrates and on the basis of their occurrence as well as evolutionary patterns they seem to display functional properties vital for the organisms [2]. Class I ADH is, in human, the 'classical' liver enzyme with high affinity for short chain alcohols, e.g. ethanol. Class III ADH, identical to glutathione-dependent formaldehyde dehydrogenase, is the most conserved enzyme among ADHs and is thought to be the ancestral form [3]. It is ubiquitously expressed and plays a crucial role as a formaldehyde scavenger in all cells. Class III ADH is highly specific for *S*-hydroxymethylglutathione (HMGSH), the natural substrate, spontaneously formed from formaldehyde and glutathione [4]. A number of conserved amino acid residues lining the substrate binding pocket have been identified to be of importance for glutathione-dependent formaldehyde dehydrogenase activity. Arg^{115} plays a central role in substrate interaction and if chemically modified or replaced by mutagenesis the catalytic efficiency of the enzyme is significantly decreased [5,6]. Asp^{57} has also been shown to be of importance for binding of HMGSH [7]. These two residues have been denominated as specificity determining since their replacement results in class III enzymes with class I-like activities [7]. Additionally, anion activation of short chain alcohol oxidation of class III ADH have been attributed to Arg^{115} [5], which electrostatically interacts with the charged anion, e.g. propanoate, and thereby makes the active site smaller and more hydrophobic. From comparison, it is obvious that through a number of residue exchanges the substrate binding pocket of class I is smaller and more hydrophobic than the pocket of class III ADH [8,9].

Here we investigate the possibility to transfer class I ADH into an enzyme with class III specific functions. Class I $\gamma\gamma$ ADH was mutated at position 57 and 115 replacing Asp for Leu and Arg for Asp, respectively, in an attempt to mimic the substrate binding pocket of class III ADH. In addition, class III ADH was mutated at position 115, exchanging Arg to Ser or Lys, in order to further investigate class III ADH substrate specificity.

2. Materials and methods

2.1. Plasmid construction and mutagenesis

Wild type and mutated forms of class III ADH were expressed in *E. coli* using constructs based on the pKK223-3 expression vector [6,7]. cDNA coding for class I $\gamma_2\gamma_2$ ADH [10] was amplified with PCR technique using *Pfu* polymerase (Stratagene) and ligated into *Nde*I and *Bam*HI digested plasmid pET12b [11] (Novagen). The R115S$\chi\chi$ mutant was engineered with mutagenesis primer 5′-CTTGAGTGACACTTATCTTCTGG-3′ using the single stranded DNA mutagenesis technique [12] according to the manufacturer's recommendations (Amersham). The R115K$\chi\chi$ and L57D/D115R$\gamma\gamma$ mutants were engineered using the unique site elimination procedure on double stranded DNA [13] with an USE Mutagenesis Kit (Pharmacia Biotech). For the R115K$\chi\chi$, 5′-CCCTTGAGTGACTTTTATCTTCTGGCA-3′ were used as mutagenesis primer and 5′-TCTCCTTGCGCGCACCATTCC-3′ as selection primer. The L57D/D115R$\gamma\gamma$ mutants were made using, 5′-TACTGCTTGAAAAATCGTCTAGGCAATCC-3′ and 5′-GGTTAGTGGCAACGACGTGACCCCCCTTC-3′ as mutagenesis primers and 5′-ACAACGGTTTCCCGCTAGAAATAATTTTG-3′ as selection primer. After mutagenesis the coding part of the double mutant, L57D/D115R$\gamma\gamma$ was subcloned into pET29a. All mutated cDNAs were verified by dideoxynucleotide sequence analysis [14]. Plasmids were isolated with the FlexiPrep Kit (Pharmacia Biotech) or the Magic Minipreps (Promega).

2.2. Protein expression and purification

Recombinant $\chi\chi$, R115S$\chi\chi$ and R115K$\chi\chi$ were expressed in 2-l cultures of *E. coli* strain TG1. Recombinant $\gamma\gamma$ and L57D/D115R$\gamma\gamma$ were expressed in 2-l cultures of *E. coli* strain BL21(DE3). In all cases, except for the L57D/D115R$\gamma\gamma$ mutant, the cells were grown over night after induction with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) at $OD_{595} \sim 1$. For isolation of recombinant protein, cells were disrupted in 10 mM Tris-HCl, 1 mM DTT, pH 8, by sonication followed by centrifugation for 1 h at $48\,000 \times g$. The supernatant, 10–20 ml, was applied to a 150-ml DEAE column (DE-52; Whatman).

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Activity containing fractions were pooled, dialyzed over night, applied to a 10-ml AMP-Sepharose column (Pharmacia Biotech) and eluted with 2.4 mM NAD^+. The proteins were concentrated by filter centrifugation (Microsep 30K cut off, Filtron) and a final purification step on gel filtration or anion-exchange chromatography on FPLC was performed. For the gel filtration a 25-ml column, Superose 12 (Pharmacia Biotech), was equilibrated with 75 ml of 50 mM sodium phosphate, 150 mM NaCl, pH 8, before the protein was applied at a flow rate of 0.3 ml/min. All recombinant proteins eluted after approximately 12 ml. In the anion-exchange chromatography a Resource Q column (Pharmacia Biotech) was used with a 30-ml linear gradient of 0–0.15 M NaCl in 10 mM Tris-HCl, pH 8, at a flow rate of 1 ml/min. Protein concentrations were determined colorimetrically [15] and the purity was analyzed by SDS-polyacrylamide gel electrophoresis. Cells expressing the L57D/D115Rγγ mutant were induced with 0.5 mM IPTG at $OD_{595} \sim 0.5$, harvested after 4 h and disrupted using the same scheme as for the wild-type enzymes. Two ml of the supernatant was immediately applied on a 20-ml DEAE column (DE-52; Whatman) and 2-ml fractions were collected. Fractions containing activity were concentrated by filter centrifugation (Microsep 30K cut off, Filtron) and used directly for kinetic characterization.

As a control, *E. coli* ADH was obtained from strain BL21 (DE3). Lysate was prepared as above and applied on a metal-chelating column (Novagen) loaded with nickel ions followed by affinity chromatography on an AMP-Sepharose column (Pharmacia Biotech). The final purification step was performed on anion-exchange chromatography column, Resource Q (Pharmacia Biotech), with a 20-ml linear gradient of 0–0.5 M NaCl in 10 mM Tris-HCl, pH 8, at a flow rate of 1 ml/min.

2.3. Immunoblot analysis and protein quantification

Crude extracts containing L57D/D115Rγγ and partially purified L57D/D115Rγγ were subjected to SDS-polyacrylamide gel electrophoresis and transferred to poly(vinylidene difluoride) transfer membrane (Bio-Rad) by electroblotting. The membrane was blocked in 5% fat-free dry milk (Semper) in 10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween 20, subsequently incubated with a 1:3000 dilution of antiserum against class I ADH, washed and finally incubated in a 1:3000 dilution of Protein A-HRP Conjugate (Bio-Rad). Immunoreactive bands were detected by ECL detection reagents according to the manufacturer's recommendations (Amersham). The bands corresponding to purified protein were scanned using a Personal Densitometer (Molecular Dynamics) and intensities were quantified and normalized to controls with known amounts of purified wild-type protein.

2.4. Enzyme kinetics

NAD^+, 12-hydroxydodecanoate (12-HDA), octanol, methylcrotyl alcohol (MCA) and propanoate were purchased from Sigma and used without further purification. Formaldehyde solutions were made from newly opened glass ampoules, 20% solutions (Ladd Research Industries). Alcohols and pentanoate were of analysis grade and all substrates were dissolved in HPLC-grade acetonitrile to yield a final concentration of 2–4% acetonitrile (v/v) in the reaction mixture, a concentration that did not affect the activity of the enzymes. Kinetic constants were determined in 0.1 mM sodium phosphate, pH 8, for HMGSH. For ethanol, octanol, 12-HDA and MCA, with or without pentanoate, two different conditions were used, 0.1 mM glycine/NaOH, pH 10, and 0.1 mM sodium phosphate, pH 7.5. Measurements were performed at 25°C, except for the L57D/115Rγγ mutant where measurements were performed at 37°C. An NAD^+ concentration of 2.4 mM was used in all experiments and the enzymatic activity was determined by following the formation of NADH using a Hitachi U-3000 spectrophotometer. A molar absorptivity of 6220 M^{-1} cm^{-1} at 340 nm was used to calculate NADH concentrations. A weighted non-linear-regression analysis program was used to calculate the kinetic constants (Fig. P for Windows; Biosoft) and k_{cat} values are based on a molecular mass of 40 kDa for all enzymes.

3. Results

3.1. Purification and yield of recombinant proteins

The two wild-type forms, class I ADH, γγ, and class III ADH, χχ, were purified to homogeneity, as judged by SDS-polyacrylamide gel electrophoresis, in three successive steps, each yielding about 2 mg pure protein/l culture. The mutant forms of class III ADH, R115Kχχ and R115Sχχ, were purified to homogeneity using the same scheme as for the wild-type forms, with a yield of approximately 0.5 mg pure protein/l culture of each. The class I ADH mutant, L57D/D115Rγγ, was very labile and yielded only about 4 μg/l culture. Therefore, only a one step purification procedure was performed and fractions had to be concentrated about 20-fold prior to kinetic characterization. Protein amounts and specific activities (with ethanol and HMGSH as substrates for class I and class III ADH, respectively) were measured after each purification step. Specific activities for the wild-type enzymes agreed well with previously published values, 1 U/mg and 4 U/mg for class I and class III ADH, respectively [7,16]. Both substitutions at position 115 gave proteins with decreased specific activities, 4-fold for R115Sχχ and 2-fold for R115Kχχ.

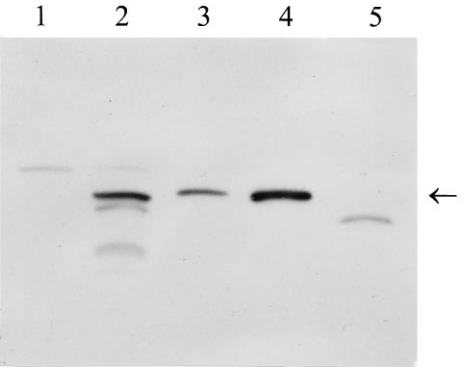

Fig. 1. Immunoblot of lysates containing L57D/D115Rγγ and purified L57D/D115Rγγ. Lane 1: Lysate from uninduced *E. coli*. Lane 2: Lysate from IPTG induced *E. coli*. Lane 3: Purified L57D/D115Rγγ. Lane 4: 200 ng purified wild-type γγ. Lane 5: 200 ng purified *E. coli* ADH. Arrow indicates signal from purified L57D/D115Rγγ and wild-type control γγ.

Immunoblot analysis of crude extracts containing L57D/D115Rγγ and purified protein showed a distinct band with the same size as control protein indicating that recombinant protein is expressed after induction with IPTG (Fig. 1). No immunoreactive band corresponding to ADH was detected without induction. Additionally, large amounts of shorter bands corresponding to peptide fragments appear after IPTG induction, most likely due to extensive degradation of unstable mutant class I protein. *E. coli* ADH can be present in lysates giving rise to undesired endogenously formed ADH activity. This protein as well as degraded class I protein is excluded by DEAE ion exchange chromatography as can be judged from Western blot analysis (Fig. 1) and SDS-polyacrylamide gel electrophoresis, resulting in one single immunoreactive band of intact L57D/D115Rγγ protein (Fig. 1).

3.2. Kinetic characterization of recombinant proteins

HMGSH oxidation activities were determined in 0.1 mM sodium phosphate, pH 8. Oxidation rates of 12-HDA, octanol, ethanol and MCA with or without pentanoate, were determined in 0.1 mM glycine-NaOH, pH 10, and 0.1 mM sodium phosphate, pH 7.5, in order to assess pH-dependent differences in activities. Generally, 5- to 15-fold lower k_{cat} values were recorded at pH 7.5 as compared to pH 10.

The wild-type class I γγ enzyme exhibited K_m and k_{cat} values at pH 10 for 12-HDA and octanol consistent with previ-

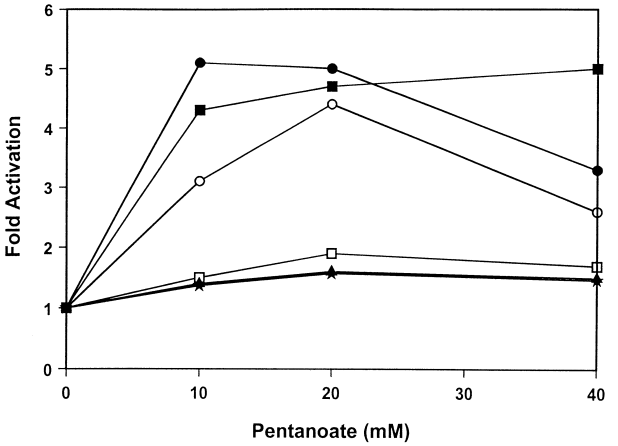

Fig. 2. Effect of pentanoate on MCA oxidation for wild-type and mutated forms of χχ. (●) Wild-type χχ at pH 7.5. (○) Wild-type χχ at pH 10. (■) R115Kχχ at pH 7.5. (□) R115Kχχ at pH 10. (▲) R115Sχχ at pH 7.5. (★) R115Sχχ at pH 10.

ously published data (Table 1) [17]. At pH 7.5, K_m for 12-HDA is 15 μM and for ethanol 280 μM. For the double mutant, L57D/D115Rγγ, kinetic constants were determined at 37°C, pH 10, due to the minute amounts of protein. L57D/D115Rγγ showed a decreased K_m value for 12-HDA, an increased K_m value for ethanol and an unchanged value for octanol, as compared to the wild-type enzyme. L57D/D115Rγγ exhibited no detectable HMGSH activity and it was not possible to accelerate MCA oxidation with addition of pentanoate.

Differences in activities for HMGSH oxidation for the class III mutants, as compared to the wild-type enzyme, were mainly due to increased K_m values, while turn-over numbers remained fairly constant. R115Kχχ showed a 70-fold increase and R115Sχχ showed 145-fold increase in K_m, yielding proteins with a significantly lower specific activity (Table 1).

For oxidation of 12-HDA and for activation of MCA oxidation, R115Kχχ displayed changes in kinetic constants that were pH-dependent. At neutral pH, 12-HDA oxidation and activation were nearly as efficient as for the wild-type enzyme, while at pH 10 marked decrease in 12-HDA affinity and almost abolished activation was monitored (Table 1 and Fig. 2). R115Sχχ showed kinetic differences regardless of pH, visualized by increased K_m values for 12-HDA and no activation of MCA oxidation at pH 7.5 and as well at pH 10. Only minor kinetic differences were recorded for octanol between the class III variants.

4. Discussion

The class I and class III (glutathione-dependent formaldehyde dehydrogenase) ADH differ considerably in substrate specificity in order to serve as detoxifiers of different alcohols and formaldehyde. Amino acid residues at positions 48, 57, 93 and 115 are all positioned in the substrate binding pocket and have been targets for mutational analysis to elucidate substrate-enzyme interactions [7,18]. Asp^{57} and Arg^{115} are invariant within class III ADH and they have been shown to be crucial for HMGSH activity [6,7]. Activities of primary alcohols such as pentanol and octanol are not affected when amino acid residues are exchanged at position 57 or 115 which have led to the denomination of these positions as specificity-determining [7]. Furthermore, crystallographic data on human class III ADH have recently endorsed the earlier hypothesis that Asp^{57} electrostatically interacts with the α-amino group of the γ-glutamyl residue and that Arg^{115} electrostatically interacts with the carboxylate group of the glycine residue in the formed HMGSH [9].

Since class III ADH has been shown to be the origin of the medium chain ADH system, the class I enzyme has evolved from class III [2,3], lost the glutathione-dependent formaldehyde dehydrogenase activity but enhanced the ethanol dehydrogenase activity. The attempt to mutate class I 'back' to class III by changing the residues at position 57 and 115, L57D/D115Rγγ, resulted, however, in a very unstable protein. Both positions are situated in the substrate binding pocket which in the class I enzyme is relatively narrow. The intro-

Table 1
Kinetic constants for recombinant wild-type and mutated enzymes at pH 7.5[a] and pH 10

Enzyme	HMGSH			12-HDA			Octanol			Ethanol		
	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1}/mM)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1}/mM)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1}/mM)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1}/mM)
pH 10												
χχ		n.d.[e]		50	60	1200	550	75	140	–	–	0.045[b]
R115Sχχ		n.d.[e]		380	80	210	470	90	190	–	–	n.d.[e]
R115Kχχ		n.d.[e]		270	120	440	550	100	180	–	–	n.d.[e]
γγ		n.d.[e]		55	40	700	5	50	10 000	560	48	85
L57D/D115Rγγ[c]		n.d.[e]		15	200	13 300	5	35	7000	2500	35	14
pH 7.5												
χχ	4[b]	200[b]	50 000[b]	80	10	125	900	10	11	–	–	n.d.[e]
R115Sχχ	580	150	260	200	9	45	750	12	16	–	–	n.d.[e]
R115Kχχ	290	155	530	80	5	65	700	4	6	–	–	n.d.[e]
γγ		n.a.[d]		15	30	2000	10	20	2000	280	34	120
L57D/D115Rγγ		n.a.[d]			n.d.[e]			n.d.[e]			n.d.[e]	

k_{cat} values are calculated per subunit with the molecular mass of 40 kDa.
[a]Except for HMGSH constants which are determined at pH 8.
[b]Values given are from [7].
[c]Measurements were performed at 37°C.
[d]No activity.
[e]Not determined.

duction of charged residues probably results in conformational changes and tensions within the structure. The K_m value observed for ethanol is five times higher for the L57D/D115Rγγ mutant than for the wild-type enzyme which might be a consequence of a widening of the inner part of the substrate pocket. For octanol, however, the affinity is hardly changed and this should be compared to the class III ADH K_m value for octanol which is 100-fold higher. Thus, the substrate pocket is probably not affected in the middle and outer part. Interestingly, the K_m value for 12-HDA is lowered nearly 5-fold, indicating possible charge interaction between the Arg at position 115 and the substrate. This is a class III specific feature and in the latter sense it is partly possible to force the class I enzyme to show class III-like characteristics. Still, HMGSH activity and hydrophobic anion activation is not detectable, most likely due to the fact that the pocket is not large enough to fit a HMGSH molecule.

For the class III mutants, R115Sχχ and R115Kχχ, significant changes in HMGSH and 12-HDA oxidation rates were observed mainly due to increased K_m values. The results for R115Sχχ are in agreement with an earlier mutation study where Arg was replaced by Ala or Asp and is explained by loss of charge interaction with the substrate [6]. Arg and Lys show chemical similarities, i.e. positive charge at neutral pH, and a 70-fold increase in K_m for R115Kχχ was not expected since the requirements for electrostatic interaction, accompanied by good substrate binding, are present. The mutated protein exhibited a specificity constant that was decreased by nearly two orders of magnitude. The reduced activities for these two mutants leads to the conclusion that not only a positive charge but also the exact positioning is of utmost importance for correct docking of HMGSH into the active site. The Lys mutant showed a strong pH dependence for 12-HDA reflecting the pK_a of Lys and again, the prerequisite for a charge interaction is visualized.

Small substrates such as ethanol and MCA are poorly oxidized by class III ADH but can be activated by the addition of hydrophobic anions, interacting electrostatically with Arg^{115} in the native enzyme [5,19]. The results for the mutants reflect the pH dependence of Lys and the non-charged nature of Ser. At pH 7.5, the R115Kχχ mutant is activated in the same range as the wild-type enzyme (Fig. 1) whereas the activation is almost abolished at pH 10. This is in line with the kinetic data obtained for 12-HDA where the pH-dependent differences in kinetics are also observed.

The physiological function of class III ADH, scavenging endogenously formed formaldehyde, is a very well preserved feature that is reflected in the conservation of the enzyme and its wide distribution throughout the kingdoms [1,3]. On the other hand, class I ADH is not as conserved and its kinetic parameters differ between the species. Even though a few residues at the substrate binding pocket of class III have been attributed to class specific functions, the introduction of corresponding residues into another class does not, evidently, result in an enzyme with overall class III specific features. The crystallographic data for class III ADH showed that Arg^{115} is positioned in an α-helix structure, not found in other human ADHs, and that Asp^{57} is positioned in a loop structure close to the catalytic zinc [9], altogether yielding a large substrate pocket. The point mutation in class I at position 115 will probably not induce this α-helix that seems to promote HMGSH binding. Furthermore, from docking experiments of HMGSH into the determined structure of class III ADH, Thr^{52} and Tyr^{140}, residues typical for class III ADH, are suggested to hydrogen bond to the substrate [9]. However, if these residues were introduced into the class I ADH mutant they would probably not enlarge the substrate binding pocket, a prerequisite to generate HMGSH binding. A cod ADH has been described as a transition form between class I and III ADH [20] and in resemblance with the human class III structure an α-helix and a loop are present at the active site pocket. Despite this, HMGSH binding is prevented by exchanges that narrow the pocket [9,20]. The point mutations in class III performed here, together with earlier results [6,7], show that the charged residues at positions 57 and 115 are essential for an active class III ADH. Results from the class I mutant indicate that an overall widening of the active site pocket, not accomplished by point mutations, is a prerequisite for HMGSH binding, thus explaining the absence of HMGSH oxidation activity in the class I mutant.

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References

[1] Jörnvall, H. and Höög, J.-O. (1995) Alcohol Alcohol 30, 153–161.
[2] Jörnvall, H., Shafqat, J., el-Ahmad, M., Hjelmqvist, L., Persson, B. and Danielsson, O. (1997) Adv. Exp. Med. Biol. 414, 281–289.
[3] Danielsson, O. and Jörnvall, H. (1992) Proc. Natl. Acad. Sci. USA 89, 9247–9251.
[4] Uotila, L. and Koivusalo, M. (1989) in: Coenzymes and Cofactors, Glutathione Chemical, Biochemical and Medical Aspects, Part A, Vol. 3 (Dolphin, D., Poulson, R. and Avramovic, O., Eds.) pp. 517–551, Wiley, New York, NY.
[5] Holmquist, B., Moulis, J.M., Engeland, K. and Vallee, B.L. (1993) Biochemistry 32, 5139–5144.
[6] Engeland, K., Höög, J.-O., Holmquist, B., Estonius, M., Jörnvall, H. and Vallee, B.L. (1993) Proc. Natl. Acad. Sci. USA 90, 2491–2494.
[7] Estonius, M., Höög, J.-O., Danielsson, O. and Jörnvall, H. (1994) Biochemistry 33, 15080–15085.
[8] Davis, G.J., Bosron, W.F., Stone, C.L., Owusu-Dekyi, K. and Hurley, T.D. (1996) J. Biol. Chem. 271, 17057–17061.
[9] Yang, Z.N., Bosron, W.F. and Hurley, T.D. (1997) J. Mol. Biol. 265, 330–343.
[10] Höög, J.-O., Hedén, L.-O., Larsson, K., Jörnvall, H. and von Bahr-Lindström, H. (1986) Eur. J. Biochem. 159, 215–218.
[11] Svensson, S., Lundsjö, A., Cronholm, T. and Höög, J.-O. (1996) FEBS Lett. 394, 217–220.
[12] Taylor, J.W., Ott, J. and Eckstein, F. (1985) Nucleic Acids Res. 13, 8765–8785.
[13] Deng, W.P. and Nickoloff, J.A. (1992) Anal. Biochem. 200, 81–88.
[14] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[15] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[16] Deetz, J.S., Luehr, C.A. and Vallee, B.L. (1984) Biochemistry 23, 6822–6828.
[17] Eklund, H. et al. (1990) Eur. J. Biochem. 193, 303–310.
[18] Höög, J.-O., Eklund, H. and Jörnvall, H. (1992) Eur. J. Biochem. 205, 519–526.
[19] Moulis, J.M., Holmquist, B. and Vallee, B.L. (1991) Biochemistry 30, 5743–5749.
[20] Ramaswamy, S., el Ahmad, M., Danielsson, O., Jörnvall, H. and Eklund, H. (1996) Protein Sci. 5, 663–671.