article II

Contribució de B.Crosas en el treball:

- Anàlisi de la interacció entre 1-pentanol i el complex binari ADH4 humana-NAD^+ mitjançant modelatge molecular i simulació de docking

ELSEVIER

Chemico-Biological Interactions

Chemico-Biological Interactions 000 (2000) 000–000
www.elsevier.com/locate/chembiont

Kinetic effects of a single-amino acid mutation in a highly variable loop (residues 114–120) of class IV ADH

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Abstract

Class IV alcohol dehydrogenase shows a deletion at position 117 with respect to class I enzymes, which typically have a Gly residue. In class I structures, Gly117 is part of a loop (residues 114–120) that is highly variable within the alcohol dehydrogenase family. A mutant human class IV enzyme was engineered in which a Gly residue was inserted at position 117 (G117ins). Its kinetic properties, regarding ethanol and primary aliphatic alcohols, secondary alcohols and pH profiles, were determined and compared with the results obtained in previous studies in which the size of the 114–120 loop was modified. For the enzymes considered, a smaller loop was associated with a lower catalytic efficiency towards short-chain alcohols (ethanol and propanol) and secondary alcohols, as well as with a higher K_m for ethanol at pH 7.5 than at pH 10.0. The effect can be rationalized in terms of a more open, solvent-accessible active site in class IV alcohol dehydrogenase, which disfavors productive binding of ethanol and short-chain alcohols, specially at physiological pH.

Keywords: Alcohol dehydrogenase; Class IV; Ethanol

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0009-2797/00/$ - see front matter
PII: S0009-2797(00)00288-X

1. Introduction

Class IV alcohol dehydrogenase (ADH) shows distinct kinetic properties, when compared with class I enzymes, such as higher K_m values for ethanol and short-chain alcohols [1,2], low activity with secondary alcohols [1,3,4], and higher K_m values for ethanol at pH 7.5 than at pH 10.0 [1,5].

The amino acid sequence of human class IV ADH differs from those of class I enzymes in about 30% of the residues [2]. Crystallographic data [6,7] and molecular models [8–10] have shown that the class IV three-dimensional structure follows the general protein fold of class I enzymes. The main structural differences with functional relevance for class IV, observed in the substrate-binding pocket, are a deletion at residue 117 (Gly in class I) and the substitution of Ala294 for a Val residue in rodent class IV enzymes. Both the deletion at 117 [4,8] and the exchange at 294 [2,11] have been pointed out to explain some of the distinct kinetic properties of class IV enzymes, and have been the subject of a recent publication [10].

In class I ADH, residue 117 is part of a loop comprising residues 114–120, which is located near the entrance of the substrate-binding pocket [12,13], within the previously defined variable segment $V2_1$ [14]. This loop restricts the access of bulky substrates to the active site and it is flexible enough to adopt different conformations in binary and ternary complexes [12,15]. The length and sequence of this loop is highly variable in ADH between different classes and species, with many substitutions, insertions and deletions [16], and it is involved in determining substrate specificity [17]. In this regard, deletion of residue 115, naturally found in the horse class I SS form, renders the enzyme active with steroids while the EE isozyme (with Asp at position 115) is inactive with these substrates [18,19]. In class III ADH, the loop becomes an α-helix, which along with Arg115 is essential for *S*-hydroxymethylglutathione binding [17,20].

In the present work, we provide further insights on the kinetic effects of a single-amino acid mutation (G117ins) in a highly variable loop of class IV ADH.

2. Materials and methods

Human class IV ADH cDNA was subjected to site-directed mutagenesis and expressed in *E. coli* strain BL21(DE3)/plysS, using a pET5a plasmid, as previously described [10]. Wild-type and mutant enzymes were purified by DEAE-sepharose and AMP-sepharose chromatographies. Except otherwise stated, alcohol dehydrogenase activity was determined at 25°C, in 0.1 M sodium phosphate, pH 7.5, in the presence of 2.4 mM NAD^+. Buffers used for pH profiles were 33 mM sodium phosphate, pH 7.0–8.0, and 100 mM glycine/NaOH, pH 9.0–11.0. At each pH, the data were fitted to the Michaelis–Menten equation by means of the ENZFITTER (Elsevier Biosoft) software. Substrate docking of 1-pentanol to the human class IV structure (PDB code: 1AGN, [6]) was performed by using the ICM program (version 2.7, Molsoft LLC, 1997), as described [10].

3. Results and discussion

3.1. Kinetics with ethanol and other primary aliphatic alcohols

Table 1 shows the K_m values for primary aliphatic alcohols of the wild-type human class IV and the G117ins mutant. Also, data on single-amino acid mutations involving loop residues 115 [19] and 116 [21] are included for comparison. In the three cases examined, the size of the loop 114–120 was substantially diminished, either by deletion (positions 115 and 117) or substitution by a residue with a smaller atomic volume (position 116).

When comparing the G117ins mutant with the wild-type class IV enzyme, a moderate increase was seen in the K_m values for ethanol and propanol (Table 1). The increase was more marked in the horse EE D115del and the human $\beta_1\beta_1$ L116A mutants, with respect to their wild-type counterparts (Table 1), probably because the change in residue volume was larger and affected the inner part of the loop. For substrates containing four or more carbons, the results were quite different. In the class IV enzyme, the K_m values decreased about 3–5-fold with respect to the G117ins mutant, while in the enzymes with changes at positions 115 and 116 the K_m values suffered a slight increase or did not change.

Catalytic constants remained relatively unchanged as the length of the carbon chain of the alcohol increased [10,19,21], and thus changes in k_{cat}/K_m values were primarily due to the decrease in K_m values (Table 1). When $\log(k_{cat}/K_m)$ was plotted against the number of carbons for a series of primary aliphatic alcohols (Fig. 1), the group of enzymes with a more extended loop (G117ins, horse EE, and human $\beta_1\beta_1$) showed linear relationships with moderately positive slopes, indicating a contribution of substrate hydrophobicity (due to apolar methylene groups in the aliphatic chain) to the binding in the active-site pocket. In contrast, the wild-type class IV and the L116A mutant displayed biphasic curves with abrupt discontinuities in the

Table 1
K_m values (mM), at pH 7.5, with primary aliphatic alcohols of wild-type and mutant ADH forms involving changes in loop residues 115, 116, and 117

Substrate	Residue 115, class I horse EE[a]		Residue 116, class I human $\beta_1\beta_1$[b]		Residue 117, class IV human $\sigma\sigma$[c]	
	Wild type[d]	D115del	Wild type[d]	L116A	G117ins[d]	Wild type
Ethanol	0.54	60	0.05	1.1	12	42
Propanol	0.17	3.6	0.02	0.9	8.7	11
Butanol	0.13	0.43	0.01	0.07	4.9	1.0
Pentanol	0.14	0.11	0.02	0.05	1.15	0.31
Hexanol	0.056	0.062	0.02	0.02	0.46	0.14

[a] Determined at 30°C, pH 7.3 [19].
[b] Ref. [21].
[c] Ref. [10].
[d] Enzymes containing a more extended loop.

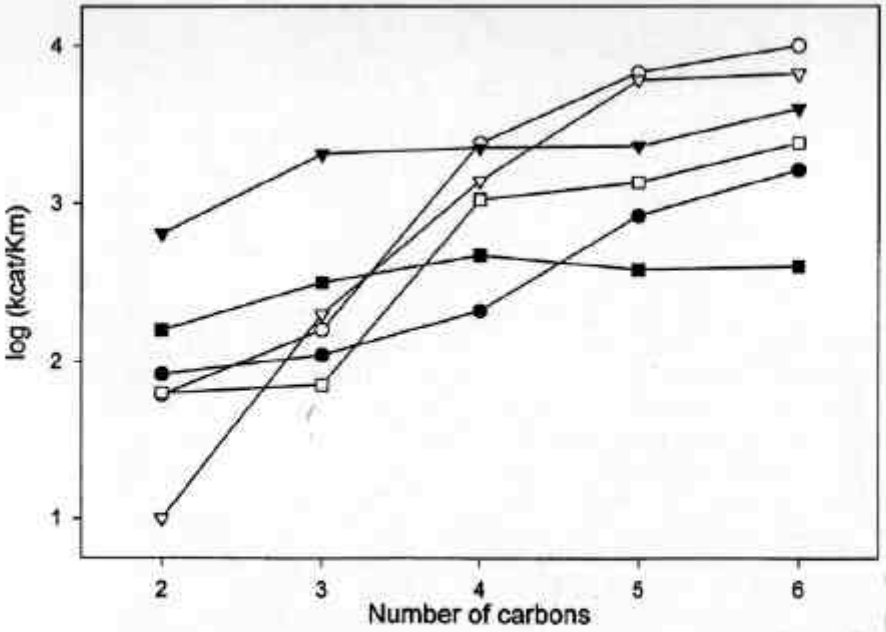

Fig. 1. Variation of catalytic efficiency as a function of substrate chain length for different ADH enzymes. All values were measured at pH 7.5, except those of EE-ADH and D115del which were determined at pH 7.3. G117ins (-●-), wild-type class IV (-○-), $\beta_1\beta_1$-ADH (-■-), L116A (-□-), EE-ADH (-▼-), D115del (-▽-).

region between three and four carbons, while the D115del mutant showed a distinct nonlinear plot. It is noteworthy that, for two- and three-carbon alcohols, the wild-type class IV and the G117ins mutant have about equal k_{cat}/K_m values (Fig. 1). However, for medium and long-chain alcohols, wild-type class IV showed the highest catalytic efficiency.

These results could reflect a poorer binding of short-chain alcohols (ethanol or propanol) and a much more favorable interaction of aliphatic alcohols having four carbons or more. In fact, a docking simulation of 1-pentanol to human class IV structure showed that residue 116 is located 6.2 Å away from the C2 atom of alcohol, and thus it would not interact with bound ethanol (Fig. 2). In contrast, C4 and C5 atoms of butanol and longer-chain substrates could interact with the loop through van der Waals contacts (Fig. 2). It is tempting to speculate that the deletion at position 117 in class IV, as well as the other amino acid substitutions discussed above, create additional space for water molecules to enter the active site, analogous to what has been proposed for the V294A mutation in class IV [10] and for class III ADH [17,22,23]. This effect could increase the number of nonproductive conformations and it would impair the correct binding of short-chain alcohols, such as ethanol, resulting in increased K_m values. As shown below, the effect of pH

on the K_m value for ethanol of the G117ins mutant, compared with the wild-type enzyme, gives further support to this hypothesis. Medium and long-chain alcohols would be less affected by the exchange at 117 because of the extra hydrophobic binding. This is consistent with the observed kinetics with octanol and retinoids [10].

3.2. Kinetics with secondary alcohols

Class IV ADH has a very low efficiency towards secondary alcohols [1,3,4] (Table 2). It had been suggested that the specificity towards secondary alcohols for class I enzymes depends on amino acid residues found at positions 48 and 93 [24]. Class IV enzymes have the same residues, Thr48 and Phe93, as $\beta_1\beta_1$-ADH, and yet their K_m values for 2-butanol isomers are three orders of magnitude higher [21] (Table 2). Deletion of Gly117 in class IV has been pointed out as responsible for this kinetic behavior [4].

The G117ins mutant showed much lower K_m values for the isomers of 2-butanol than the wild-type enzyme, in contrast to what was observed for the primary alcohol isomer 1-butanol (Table 2). Again, wild-type class IV behavior resembled that of the L116A mutant, with very high K_m values for (*S*)-2-butanol and (*R*)-2-butanol [21]. However, the K_m values were always higher for the class IV than for the class I enzymes [24].

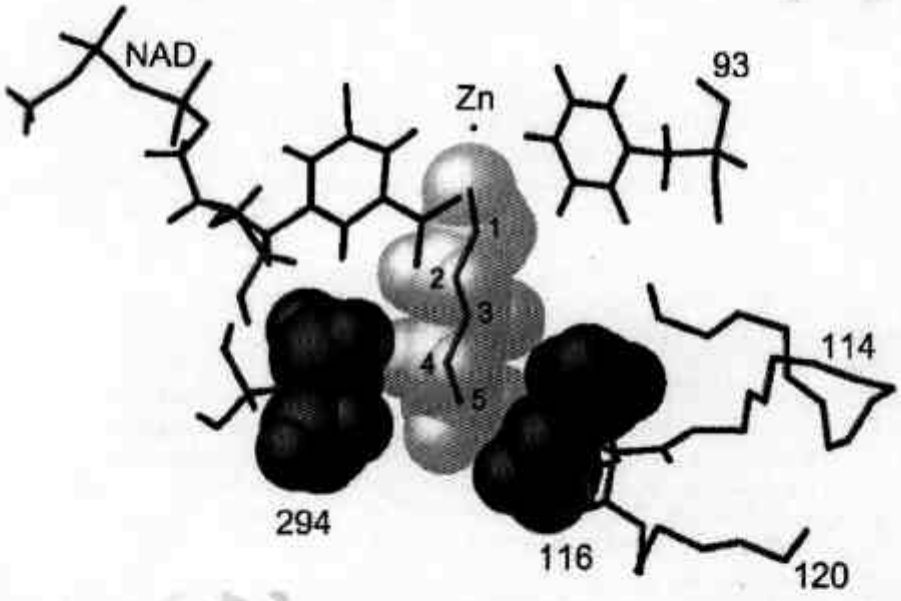

Fig. 2. Docking of 1-pentanol to the substrate-binding pocket of human class IV ADH. The van der Waals radii are represented for the substrate (in grey) and residues Ile116 and Val 294 (in black). The polypeptide backbone is shown for the loop 114–120.

Table 2
Kinetic constants of wild-type and mutant human class IV ADH with secondary alcohols

Substrate	Wild type			G117ins		
	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1}min^{-1}$)	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1}min^{-1}$)
1-Butanol	1	2400	2400	4.9	1040	210
(*R*)-2-Butanol	1200	465	0.39	49	31	0.63
(*S*)-2-Butanol	750	410	0.55	62	52	0.84
Cyclohexanol	40	1900	48	53	620	12

The k_{cat} values also showed a significant decrease, which rendered no significant differences in terms of catalytic efficiency between the class IV and the G117ins mutant. In both enzymes, though, the catalytic efficiency was far below that of class I ADH [21]. This means that the deletion at position 117, although it contributes significantly, it is not the only structural determinant that accounts for the low efficiency of class IV towards secondary alcohols.

In contradiction to a previous report [4], the wild-type enzyme was found to be active with cyclohexanol. In contrast to what happened with the 2-butanol isomers, the K_m value for cyclohexanol was less affected by the G117ins (or L116A) exchange (Table 2). This could reflect the more hydrophobic character of cyclohexanol and the larger atomic distance between the hydroxyl group and the more distal carbon in cyclohexanol in comparison to 2-butanol. This could provide additional van der Waals interactions in the substrate-binding pocket. Despite the activity with cyclohexanol, and the enlarged entrance to the active-site pocket in class IV, no activity with steroids has been reported for this enzyme [4].

3.3. Variation of kinetic constants as a function of pH

Fig. 3 displays the variation of kinetic constants of the wild-type human class IV and the G117ins mutant as a function of pH. In ADH, the pH dependence of k_{cat}/K_m is usually ascribed to ionizations of the binary complex (enzyme–NAD^+), while the pH dependence of k_{cat} depends on ionizations of the ternary complex (enzyme–NAD^+–alcohol). The pH dependence of K_m may reflect ionizations of both binary and ternary complexes [25,26]. In Fig. 3, the pH profiles of the wild-type class IV enzyme appeared to be shifted towards higher pH values with respect to those of the G117ins mutant. Although the actual pK_a values were not calculated, it could be estimated that they increased in wild-type class IV with respect to the G117ins mutant. In class I enzymes, the pK_a values responsible for

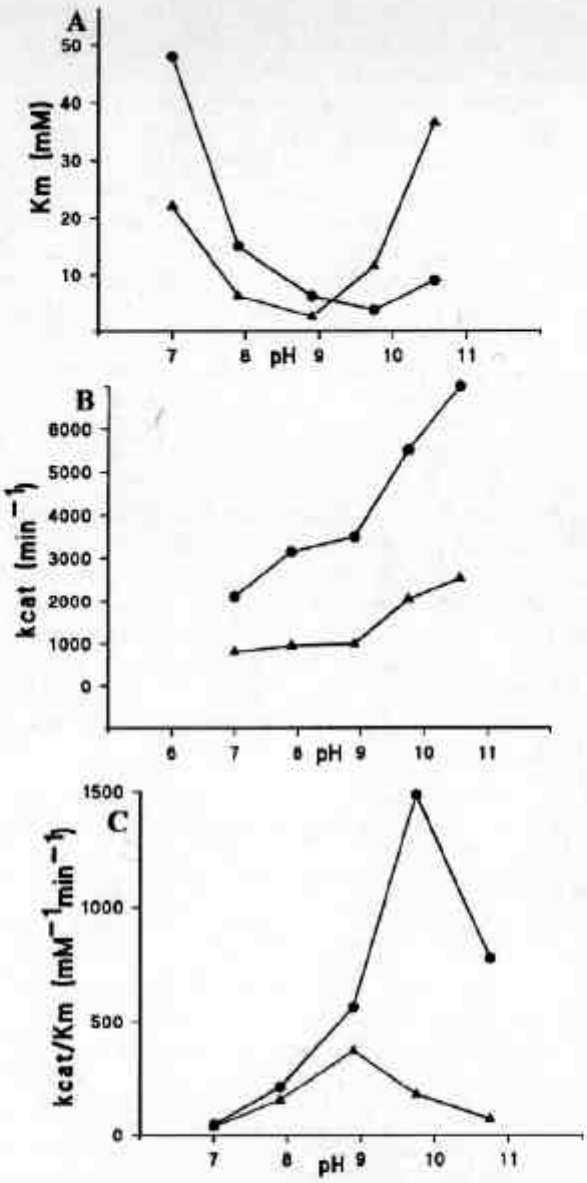

Fig. 3. Variation of kinetic constants of wild-type human class IV and G117ins mutant for ethanol oxidation as a function of pH. (A) K_m against pH; (B) k_{cat} against pH; (C) k_{cat}/K_m against pH. Wild-type (-●-), G117ins mutant (-▲-).

the pH have been assigned to zinc-bound alcohol/alcoholate or the zinc-bound water [26–28].

As previously observed for stomach class IV [1,5], the K_m values of the recombinant wild-type enzyme for ethanol decreased markedly, from 42 to 6 mM, when the pH was increased from 7.5 to 10.0 (Fig. 3A). Particularly, the dramatic 4-fold decrease in the K_m value of wild-type class IV between pH 7.0 and 8.0 could account for the differences between K_m values at pH 7.5 for human class IV reported in the literature [1,2,4,5]. Conceivably, small differences in the adjusted pH of the assay buffers could produce a significant experimental variation in K_m values.

For the G117ins mutant, the K_m for ethanol versus pH profile appeared to be shifted towards lower pH values, with a minimum value at pH 9.0 (Fig. 3A). This resulted in a K_m value slightly higher at pH 10.0 that at pH 7.5, which was just opposite to what was observed for the wild-type enzyme. Interestingly, this behavior resembles that of class I enzymes [3,29,30] and, in general, of those enzymes with a more extended 114–120 loop (Table 3). In this regard, the horse SS isozyme [30], which among other changes exhibits a deletion at position 115, follows a pattern similar to that of class IV.

The pH effect was even more marked in the V294A mutant and in the rat class IV enzyme (Table 3), both with a highly solvent-accessible active site [10]. For the rat enzyme, the K_i value for 4-methylpyrazole was also much higher at pH 7.5 than at pH 10.0 (10 vs. 0.2 mM) [2,3]. Moreover, the pH effect is attenuated as the chain length of the aliphatic alcohol increases [1,3], probably due to the increasing importance of additional hydrophobic interactions for substrate binding. Thus, a more open substrate-binding site in class IV ADH [6], accounts for a shift in pK_a values of alcohol- or water-bound zinc, with the net result of much higher K_m values for ethanol at physiological pH.

Table 3
Variation of K_m values with ethanol as a function of pH for different forms of class IV and class I ADH

Enzyme	pH 7.5	pH 10.0
Class IV human	42	6
Class IV V294A	3900[a]	280
Class IV rat[b]	2400	340
Class I horse SS[c]	22[d]	7
G117ins	9	12
Class I $\beta_1\beta_1$[e]	0.049	1.6
Class I rat[f]	1.4	1.4
Class I horse EE[c]	0.25[g]	2.0

[a] Ref. [10].
[b] Ref. [2].
[c] Ref. [30].
[d] Determined at pH 7.6.
[e] Ref. [29].
[f] Ref. [3].
[g] Determined at pH 8.0.

In conclusion, single-residue exchanges in a highly variable and flexible loop affect kinetic constants for primary aliphatic alcohols, secondary alcohols, and pH profile, in a manner consistent with a more solvent-exposed active site in the enzymes with a smaller loop. This study delineates the importance of enzyme loops in determining kinetic properties and substrate specificity, as it has been shown recently in other ADH studies [10,28] and in the aldo–keto reductase superfamily [31]. Highly variable loops in a common structural scaffold may provide the flexibility that each enzyme in a family needs to fulfill its function.

Acknowledgements

This work was supported by Grant BIO4-CT97-2123 from the Commission of the European Union, and Grants PM96-0069 and PB98-0855 from the Spanish Dirección General de Enseñanza Superior e Investigación Científica.

References

[1] A. Moreno, X. Parés, Purification and characterization of a new alcohol dehydrogenase from human stomach, J. Biol. Chem. 266 (1991) 1128–1133.
[2] J. Farrés, A. Moreno, B. Crosas, J.M. Peralba, A. Allali-Hassani, L. Hjelmqvist, H. Jörnvall, X. Parés, Alcohol dehydrogenase of class IV (σσ-ADH) from human stomach: cDNA sequence and structure/function relationships, Eur. J. Biochem. 224 (1994) 549–557.
[3] P. Juliá, J. Farrés, X. Parés, Characterization of three isoenzymes of rat alcohol dehydrogenase. Tissue distribution and physical and enzymatic properties, Eur. J. Biochem. 162 (1987) 179–189.
[4] N.Y. Kedishvili, W.F. Bosron, C.L. Stone, T.D. Hurley, C.F. Peggs, H.R. Thomasson, K.M. Popov, L.G. Carr, H.J. Edenberg, T.-K. Li, Expression and kinetic characterization of recombinant human stomach alcohol dehydrogenase, J. Biol. Chem. 270 (1995) 3625–3630.
[5] C.L. Stone, H.R. Thomasson, W.F. Bosron, T.-K. Li, Purification and partial amino acid sequence of high activity human stomach alcohol dehydrogenase, Alcohol. Clin. Exp. Res. 17 (1993) 911–918.
[6] P. Xie, S.H. Parsons, D.C. Speckhard, W.F. Bosron, T.D. Hurley, X-ray structure of human class IV σσ alcohol dehydrogenase: structural basis for substrate specificity, J. Biol. Chem. 272 (1997) 18558–18563.
[7] P. Xie, T.D. Hurley, Methionine-141 directly influences the binding of 4-methylpyrazole in human σσ alcohol dehydrogenase, Protein Sci. 8 (1999) 2639–2644.
[8] A. Moreno, J. Farrés, X. Parés, H. Jörnvall, B. Persson, Molecular modelling of human gastric alcohol dehydrogenase (class IV) and substrate docking: differences towards the classical liver enzyme (class I), FEBS Lett. 395 (1996) 99–102.
[9] M.H. Foglio, G. Duester, Molecular docking studies on interaction of diverse retinol structures with human alcohol dehydrogenases predict a broad role in retinoid ligand synthesis, Biochim. Biophys. Acta 1432 (1999) 239–250.
[10] B. Crosas, A. Allali-Hassani, S.E. Martínez, S. Martras, B. Persson, H. Jörnvall, X. Parés, J. Farrés, Molecular basis for differential substrate specificity in class IV alcohol dehydrogenases: a conserved function in retinoid metabolism but not in ethanol oxidation, J. Biol. Chem. 275 (2000) 25180–25187.
[11] X. Parés, E. Cederlund, A. Moreno, L. Hjelmqvist, J. Farrés, H. Jörnvall, Mammalian class IV alcohol dehydrogenase (stomach alcohol dehydrogenase): structure, origin, and correlation with enzymology, Proc. Natl. Acad. Sci. USA 91 (1994) 1893–1897.

[12] T.D. Hurley, W.F. Bosron, J.A. Hamilton, L.M. Amzel, Structure of $\beta_1\beta_1$ alcohol dehydrogenase: catalytic effects of non-active-site substitutions, Biochemistry 88 (1991) 8149–8153.
[13] S. Ramaswamy, M. El Ahmad, O. Danielsson, H. Jörnvall, Crystal structure of cod liver alcohol dehydrogenase: structure pocket and structurally variable segments, Protein Sci. 5 (1996) 663–671.
[14] O. Danielsson, S. Atrian, T. Luque, L. Hjelmqvist, R. Gonzalez-Duarte, H. Jörnvall, Fundamental molecular differences between alcohol dehydrogenase classes, Proc. Natl. Acad. Sci. USA 91 (1994) 4980–4984.
[15] T.D. Hurley, W.F. Bosron, C.L. Stone, L.M. Amzel, Structures of three human beta alcohol dehydrogenase variants. Correlations with their functional differences, J. Mol. Biol. 239 (1994) 415–429.
[16] H. Jörnvall, J.O. Höög, Nomenclature of alcohol dehydrogenases, Alcohol Alcohol. 30 (1995) 153–161.
[17] Z.-N. Yang, W.F. Bosron, T.D. Hurley, Structure of human $\chi\chi$ alcohol dehydrogenase: a glutathione-dependent formaldehyde dehydrogenase, J. Mol. Biol. 265 (1997) 330–343.
[18] D.H. Park, B.V. Plapp, Isoenzymes of horse liver alcohol dehydrogenase active on ethanol and steroids: cDNA cloning, expression, and comparison of active sites, J. Biol. Chem. 266 (1991) 13296–13302.
[19] D.H. Park, B.V. Plapp, Intersonversion of E and S isoenzymes of horse liver alcohol dehydrogenase: several residues contribute indirectly to catalysis, J. Biol. Chem. 267 (1992) 5527–5533.
[20] M. Estonius, J.-O. Höög, O. Danielsson, H. Jörnvall, Residues specific for class III alcohol dehydrogenase: site-directed mutagenesis of the human enzyme, Biochemistry 33 (1994) 15080–15085.
[21] T.D. Hurley, D.L. Vessell, The role of leucine 116 in determining substrate specificity in human β_1 alcohol dehydrogenase, Adv. Exp. Med. Biol. 372 (1995) 321–325.
[22] P. Julià, X. Parés, H. Jörnvall, Rat liver alcohol dehydrogenase of class III: primary structure, functional consequences and relationships to other alcohol dehydrogenases, Eur. J. Biochem. 172 (1988) 73–83.
[23] T.D. Hurley, C.G. Steinmetz, P. Xie, Z.-N. Yang, Three-dimensional structure of human alcohol dehydrogenase isoenzymes reveal the molecular basis for their functional diversity, Adv. Exp. Med. Biol. 414 (1997) 291–302.
[24] C.L. Stone, T.-K. Li, W.F. Bosron, Stereospecific oxidation of secondary alcohols by human alcohol dehydrogenases, J. Biol. Chem. 264 (1989) 11112–11116.
[25] K.F. Tipton, H.B.F. Dixon, Effects of pH on enzymes, Methods Enzymol. 63 (1979) 183–234.
[26] M.W. Makinen, W. Maret, M.B. Yim, Neutral metal-bound water is the base catalyst in liver alcohol dehydrogenase, Proc. Natl. Acad. Sci. USA 80 (1983) 2584–2588.
[27] T. Ehrig, T.D. Hurley, H.J. Edenberg, W.F. Bosron, General base catalysis in a glutamine for histidine mutant at position 51 of human liver alcohol dehydrogenase, Biochemistry 30 (1991) 1062–1068.
[28] S. Ramaswamy, D.H. Park, B.V. Plapp, Substitutions in a flexible loop of horse liver alcohol dehydrogenase hinder the conformational change and unmask hydrogen transfer, Biochemistry 38 (1999) 13951–13959.
[29] W.F. Bosron, L.J. Magnes, T.-K. Li, Kinetic and electrophoretic properties of native recombined isoenzymes of human liver alcohol dehydrogenase, Biochemistry 22 (1983) 1852–1857.
[30] C.N. Ryzewski, R. Pietruszko, Kinetic mechanism of horse liver alcohol dehydrogenase SS, Biochemistry 19 (1980) 4843–4848.
[31] M. Hasching, T.M. Penning, Conversion of mammalian 3α-hydroxysteroid dehydrogenase to 20α-hydroxysteroid dehydrogenase using loop chimeras: changing specificity from androgens to progestins, Proc. Natl. Acad. Sci. USA 96 (1999) 11161–11166.