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Variation in alcohol dehydrogenase of Drosophila

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Summary

The enzyme alcohol dehydrogenase (ADH) from *Drosophila* has been studied, for many years, by evolutionary biologists and population geneticists. These studies were mainly focused on why and how polymorphism of genes occurs in populations. The genetic variation in natural populations was primarily investigated by native polyacrylamide gel electrophoresis, which detects charge differences of amino acid sequences of proteins. Alcohol dehydrogenase of naturally occurring *Drosophila melanogaster* variants and *Drosophila simulans* ADH differ only by a few amino acids. *D. melanogaster* species can be found in habitats where alcohol concentrations are relatively high, whereas *D. simulans* does not tolerate such environments. *D. melanogaster* ADH-S and ADH-F, two widespread cosmopolitans, differ only at amino acid position 192. A thermostable variant *D. melanogaster* ADH-71k has an additional amino acid substitution at position 214 (proline to serine). *D. simulans* ADH differs from *D. melanogaster* ADH-S only at positions 1 and 82. These single or double amino acid substitutions are responsible for differences in biochemical properties. In this thesis the variation in alcohol dehydrogenase of these two sibling species is described. Although no three-dimensional structure of alcohol dehydrogenase from *Drosophila* is known, the knowledge of kinetics and the structure-function relationships should contribute to the characterization of this enzyme. Chapter 1 presents a general review of the literature of *Drosophila* alcohol dehydrogenase.

Drosophila ADH belongs to the short-chain dehydrogenases/reductases (SDR), a group of enzymes with quite different substrate specificities. The three-dimensional structures of six short-chain dehydrogenases/reductases have been elucidated recently and it was shown that, despite a low sequence homology, similarity between the three-dimensional structures was striking. In Chapter 2 we performed alignment and modelling studies with *Drosophila* ADH (DADH) and four homologous enzymes belonging to the SDR family with known X-ray structures. The coenzyme binding region is located in the N-terminal part of the enzymes, whereas the substrate binding region is located in the C-terminal one. A conserved tyrosine and lysine residue together with an almost conserved serine 139, play an important role in catalysis. This Ser-Tyr-Lys triad is found in almost all short-chain dehydrogenases/reductases and suggests that these enzymes share a common catalytic mechanism. There is little sequence homology in the substrate binding region of short-chain dehydrogenases/reductases, but a substrate-binding loop, present in most enzymes, is responsible for conformational changes upon substrate binding. This variable

loop region comprises one or two short α-helices and is located in an otherwise quite well conserved part of the structure. Model building of the DADH sequence in this substrate-binding loop suggests that in DADH these two helices may also be present. The presence of position 192, where *Drosophila melanogaster* ADH-S and ADH-F differ in sequence, at a central position in this loop is an intriguing feature.

Chapter 3 describes the purification methods used for *Drosophila melanogaster* and *Drosophila simulans* alcohol dehydrogenase. In the purification method for these enzymes affinity chromatography with the ligand Cibacron Blue is used. Introduction of a second Cibacron Blue affinity chromatography step with gradient elution with NAD^+ was necessary to obtain pure enzymes. *D. melanogaster* ADH-S was purified in a slightly different way, because it could not be removed from the affinity column using high concentrations NAD^+. Instead a wash step with a high concentration NAD^+ followed by a pH-gradient was used to obtain the enzyme. It has been reported in the literature that in homogeneous enzyme preparations, proteolytic degradation products appear within a short period of time. However, in our enzyme preparations no proteolytic degradation was found. *D. melanogaster* ADH-71k and ADH-S have a higher thermostability than ADH-F and *D. simulans* ADH.

Inhibition experiments with free ligand Cibacron Blue showed that *D. melanogaster* ADH-F and ADH-71k have identical inhibition constants (K_i) at pH 8.6 which are two times higher at pH 9.5. The K_i values for *D. simulans* ADH are three times lower at both pH values. *D. melanogaster* ADH-S also showed a low K_i value at pH 8.6, but a relatively high one at pH 9.5. Conditions required for elution of *Drosophila* alcohol dehydrogenases from the affinity column are in reasonable agreement with K_i values determined for Cibacron Blue and can be explained from amino acid replacements between the variants. The pH dependence of the properties of *D. melanogaster* ADH-S can be explained by interaction of a positive charged lysine at position 192 with NAD^+, after closure of a substrate-binding loop. The Michaelis-Menten constants (K_m) for ethanol and NAD^+ were determined for *D. melanogaster* alleloenzymes and *D. simulans* alcohol dehydrogenase and showed to be almost identical for ethanol, but the K_m^{NAD} values differ.

In Chapter 4 we show that it is possible to isolate ADH-FS from heterozygote *Drosophila melanogaster* FS flies by affinity chromatography on Cibacron Blue, using first a high concentration NAD^+, immediately followed by a pH-gradient. This isolation had to be performed at room temperature, because

when it was performed at 4 °C it was not possible to separate ADH-FS from ADH-SS and ADH-FF. The inhibition constant of *D. melanogaster* ADH-FS for Cibacron Blue, at pH 8.6 is very similar to that of ADH-SS. However, at pH 9.5 the K_i value is only slightly higher, and now much lower than those of ADH-FF and ADH-SS. Although there is no cooperativity in inhibitor binding, the two subunits in *D. melanogaster* alcohol dehydrogenase influence each other. Negative cooperativity is observed in kinetic experiments on *D. melanogaster* ADH-FS. This can be explained by the presence of binding sites with different substrate affinities so that the stronger binding site is occupied first.
Dissociation *in vitro* of *D. melanogaster* ADH-FF and ADH-SS homodimers occurs during incubation in the presence of 2 to 3 M urea for 30 minutes at room temperature. After removal of urea it is possible to obtain stable ADH-FS hybrids by reassociation of the monomers.

In Chapter 5 the substrate- and stereospecifities for primary and secondary alcohols for *Drosophila melanogaster* ADH-F and *Drosophila simulans* alcohol dehydrogenase are described. The substrate specificity for *D. simulans* ADH showed that the highest activity was obtained with 1-pentanol (primary alcohols) and 2-hexanol (secondary alcohols). However, the activity for primary and secondary alcohols is lower than compared to other *Drosophila* species. *Drosophila* alcohol dehydrogenase operates by an ordered ternary-complex mechanism with coenzyme (NAD^+) as the leading substrate. The rate limiting step for secondary alcohols in this mechanism is dissociation of NADH from the final enzyme-NADH complex. In the presence of a high concentration NAD^+ and high concentrations secondary alcohols substrate activation is observed for *D. simulans* ADH. When substrate activation occurs for *D. simulans* ADH, dissociation of NADH from the ternary enzyme-NADH-sec.alcohol complex becomes the rate limiting step. The K_m values for substrate activation are higher, indicating a lower affinity for secondary alcohols of the *D. simulans* ADH-NADH complex than of the *D. simulans* ADH-NAD^+ complex.
Stereospecificity experiments with R(-) and S(+) enantiomeric forms of secondary alcohols show that *Drosophila simulans* alcohol dehydrogenase, like other *Drosophila* alcohol dehydrogenases prefers the R(-) enantiomeric forms of secondary alcohols. From these studies it can be concluded that the alcohol binding region has room for a small and a large hydrophobic side chain of the substrate.

Drosophila alcohol dehydrogenase can be converted *in vitro* by incubation with NAD^+ and ketone to a more negatively charged isoform (Chapter 6). Although being inactive, the isoforms showed

activity after native polyacrylamide gel electrophoresis, but this depends on the ketone used. Responsible for the more negatively charged isoforms is the formation of a covalently bound adduct between NAD^+ and ketone. In chapter 6 the isolations of ADH isoforms and of the negatively charged adducts obtained in the absence of enzyme by ion-exchange FPLC on a MONO-Q column are described. When ADH is incubated with NAD^+ and acetylacetone, isoforms appear which have a larger mobility than the modified isoforms observed with other ketones. Thermal denaturation and proteolytic degradation of modified ADH isoforms resulted in release of the adduct. Thermal denaturation experiments also showed that modified ADH isoforms are more stable than the unmodified form as a result of closure of this loop upon substrate binding.