Beilstein-Institut

ESCEC, March 19th – 23rd, 2006, Rüdesheim/Rhein, Germany

Kinetic Characterization of Alcohol Dehydrogenases and Matrix Metalloproteinases: A Reflection on Standardization of Assay Conditions

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Received: 3rd July 2006 / Published: 31st August 2007

Abstract

The present paper will focus on the characterization of enzymes from two different types of family, Short Chain Dehydrogenases/Reductases and Matrixins. The former family includes over 3000 enzymes, and I have worked mainly with different allelic variants of alcohol dehydrogenase (ADH) from the fruit fly *Drosophila melanogaster* and the ADH in *Drosophila lebanonensis*. To date, approximately 25 matrix metalloproteinases are known in humans. I will focus here on both similarities and differences in problems regarding the standardization of assay conditions and parameters that I have experienced during my work with these two different enzyme systems.

Introduction

The biochemical characterization of enzymes requires careful and well planned experimental set-ups. Among parameters that need to be considered are the type of buffer to be used, what pH value is relevant to use, the ionic strength of the assay, are additives necessary, relevant temperature and what type of assay can be used in kinetic characterizations. Enzymes vary in their *in vivo* localization, their interactions with other proteins and cellular components that may affect their stability as well as their biological activity. By purification an enzyme is removed from its environment, which results in that some

enzymes need additives to compensate for the loss of interaction partners. This of course creates a problem with respect to standardization of enzyme assay conditions, which were nicely described by Tipton and co-workers [1] in the 2003 meeting on Experimental Standard Conditions of Enzyme Characterizations. In the present paper, I will focus therefore on two problems that frequently occur in the literature with respect to standardization. The first problem concerns the determination of enzyme concentration used to calculate kinetic coefficients. The second problem concerns the use of additives that have an effect on the biochemical parameter studied and to what extent the description of experimental conditions is sufficient to reproduce reported results. I will elucidate these problems mainly from my own work with two different enzyme systems, alcohol dehydrogenase from *drosophila* (DADH) and matrix metalloproteinases (MMPs). First, the two enzyme systems will be briefly described, and thereafter I will continue with the standardization problems.

Drosophila Alcohol Dehydrogenase

The ADH (EC 1.1.1.1) from insects is involved in the metabolism of short and medium sized primary and secondary alcohols, which is converted to their corresponding aldehydes and ketones (Equation 1), using the coenzyme NAD^+ [2]. The ADH is also involved in the oxidation of the formed aldehydes to their corresponding carboxylic acids (Equation 2) [3,4].

$$\text{alcohol} + NAD^+ \leftrightarrow \text{aldehyde/ketone} + NADH + H^+ \qquad (1)$$

$$\text{aldehyde} + H_2O + NAD^+ \leftrightarrow \text{carboxylate}^- + NADH + 2\,H^+ \qquad (2)$$

ADH has been found in most of the *drosophila* species investigated, and some of these species are polymorphic with respect to the Adh gene such as *D. melanogaster*, while other species such as *D. lebanonensis* are monomorphic [5].

The insect ADHs differ from the well known ADHs from other species such as vertebrates and plants in that it lacks metal ions and has a much shorter polypeptide chain [6, 7]. At the beginning of the 1980 s Jörnvall and colleagues used these differences to divide the dehydrogenases into families [7] and today, over 3000 open reading frames has been detected for the family of Short-Chain Dehydrogenases/Reductases (SDR), the family to which DADH belongs [8]. Enzymes belonging to the SDR family have been found in all species from humans to viruses [8] and they involve various enzyme classes such as oxidoreductases, lyases and isomerases. Structurally the SDR enzymes differ from the other families of dehydrogenases and reductases in that they are one domain enzymes where the N-terminal part of the polypeptide chain builds up the coenzyme binding region and the C-terminal part the catalytic region [6, 8]. It was first in 1998 that the first 3D structure of a DADH was reported, and later on followed by binary DADH–coenzyme and ternary DADH–coenzyme–substrate/product complexes [9–11]. Many studies on DADH have been

performed in order to understand the evolution and the metabolic function of this enzyme [5, 12]. DADHs have also been characterized with respect to substrate specificity, coenzymes and substrate stereospecificity, inhibitory kinetics, reaction mechanism, pH and temperature dependence, and interconversion of electrophoretic variants [2, 13–19].

Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) is the name of a group of enzymes either secreted into the extracellular matrix (ECM) or bound to the cell membrane that together are able to degrade almost all the structural ECM proteins as well as several non-ECM proteins [20]. MMPs belong to the Clan MA, subclan MAM, family M10, subfamily A (Merops database) [21]. Typical for MMPs is that they are zinc and calcium dependent. They contain two zinc ions, one catalytic and one structural. Calcium is necessary both for the stability and the activity of these enzymes [20]. Based on the substrate specificity, similarities in the primary structure and organization of the protein domains, the MMPs can be divided into six classes, matrilysins, collagenases, gelatinases, stromelysins, membrane-type MMPs and others/new MMPs [22, 23]. The general domain structure of MMPs is shown in Fig. 1 along with the structure of the different classes of MMPs. Most MMPs contain an N-terminal signal and pro-domain, a catalytic domain containing the catalytic zinc ion, a hinge domain and a C-terminal hemopexin like domain. In four of the six membrane-type MMPs (MT-MMPs), the C-terminal domain ends in a type I transmembrane domain, while two binds to the cell membrane through a glycosyl-phosphatidyl-inosityl (GPI) anchor. Two other potential MT-MMPs (MMP-23A and B, which have the same primary structure, but are coded by two different genes) contain a type II transmembrane domain (signal anchor) N-terminal to the pro-domain, and instead of a hemopexin domain they contain a unique “cystein-array” and an immunoglobulin-like (Ig) domain. The two gelatinases (MMP-2 and MMP-9) also contain a fibronectin II-like insert in their catalytic domains, while the hinge-region of MMP-9 also contains a collagen V-like domain.

What these enzymes have in common is that most are synthesized and secreted into the extracellular tissues as inactive proenzymes that need to be activated. ProMMPs can be activated by other proteinases including active MMPs in the tissues or on the cell membrane, by chaotropic agents, organomercurials, reactive oxygen species or oxidized glutathione [24]. Due to the unique sequence (RX[K/R]R) in the end of the pro-domain of the MT-MMPs, MMP-11, -21 and -28 these enzymes can be activated intracellularily by furin, a serine proteinase that belongs to the convertase family [22–24]. The activity of MMPs is also regulated by endogeneous inhibitors such as α2-macroglobulin and the specific tissue inhibitors of MMPs (TIMPs) [22, 23, 25].

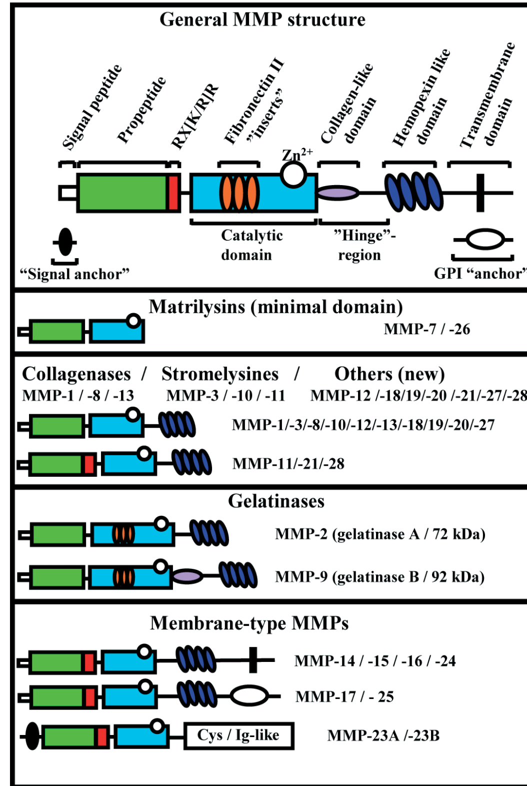


Figure 1. Schematic representation of the domain structure of MMPs. The general domain structure of MMPs is shown (top) along with the individual human MMPs that are classified according to their substrate specificity, similarities in the primary structure and organization of the protein domains.

Detection of Kinetic Coefficients Requires that the Amount of Functional Enzyme Active Sites is Determined

To get a full description of an enzyme and its ability to act on various substrates, it is necessary to determine the kinetic coefficients with substrates, coenzymes and other factors that are involved in the reaction. Equations 3 and 4 are examples of nomenclature for a two substrate reaction where *S* and *C* represent substrate and coenzyme, respectively.

$$\frac{e}{v} = \phi_0 + \frac{\phi_1}{[C]} + \frac{\phi_2}{[S]} + \frac{\phi_{12}}{[C][S]} \qquad (3)$$

$$\frac{1}{v}=\frac{1}{V_m}+\frac{K_{m1}}{V_m[C]}+\frac{K_{m2}}{V_m[S]}+\frac{K_{m2}K_{ia}}{V_m[C][S]} \quad (4)$$

$$\frac{e}{v}=\frac{1}{k_{cat}}+\frac{K_{m1}}{k_{cat}[C]}+\frac{K_{m2}}{k_{cat}[S]}+\frac{K_{m2}K_{ia}}{k_{cat}[C][S]} \quad (5)$$

Independent of nomenclature, to obtain a full description of the kinetic coefficients the concentration of functional enzyme active sites is required. As can be seen from the above described examples of nomenclature, in Equation 3 [26] the enzyme concentration is incorporated in the rate equation while this is not the case in equation 4 [27]. In the latter case it is necessary to convert V_m to k_{cat}, i.e. the coefficient for the catalytic centre activity of the enzyme. As $k_{cat} = V_m/[e]$, Equation 4 can be rewritten to Equation 5. With knowledge of k_{cat} it is possible to get a description of the enzymes capability to act on a substrate, and also to compare the activity with other similar enzymes.

A large problem is to find a good and reliable method to determine the amount of functional enzyme active sites in order to calculate k_{cat} ($1/N_0$). In the literature, it can often be seen that the amount of enzyme used in the calculations is not based on a reliable method that determines the concentration of functional active sites. Instead, the amount of protein is determined by a protein detection method such as Bradford, or A_{280nm} and a well defined extinction coefficient for the enzyme in question. Even if the enzyme preparation can be regarded to be homogeneous based on SDS-PAGE and isoelectric focusing, none of these methods are acceptable to determine the amount of functional enzyme. The reason is that these methods are based on the assumption that the protein concentration is identical with the concentration of functional active sites in the enzyme, which is not always the case. Therefore, a reliable value for k_{cat} ($1/N_0$) can be obtained only if the amount of functional enzyme is determined by a method that is based on active-site titration. How the titration is performed depends on the enzyme, and several methods have been described [28]. A good example of active site titration of ADHs was first shown by Theorell [29], which was based on the formation of a dead end ternary complex using the alcohol competitive inhibitor pyrazole. This method has been used in several studies of ADHs [30–32]. Unfortunately not all ADHs form a strong ternary complex with pyrazole, which is a necessity for its use as a titrating agent [33]. Under such conditions, it is necessary to find alternative methods. The method of Theorell [29] has been used on sorbitol dehydrogenase (SDH) from sheep liver, where DTT (a substrate competitive inhibitor) was used instead of pyrazole [34]. Several titration methods have been used on proteinases, including the classical titration method of chymotrypsin [28]. With MMPs, various methods have been used. All are based on a strong interaction between a synthetic inhibitor or one of the TIMPs and the enzyme active site [35–37]. Here of course it is important not to use a TIMP that is known to bind to a proMMPs C-terminal hemopexin-like region such as TIMP-2 to proMMP-2 and TIMP-1 to proMMP-9 [25].

The question is whether it is correct or not to report a kinetic coefficient such as k_{cat} and k_{cat}/K_{m} for a substrate when a homogeneous enzyme preparations has been used and where it is not possible to obtain the amount of functional enzyme by an active site titration method. Personally I think this is wrong, even if it is a good reason to assume that the concentration of functional active-sites is identical with the amount of enzyme detected with for example $A_{280\mathrm{nm}}$ and a well defined extinction coefficient for the enzyme in question. In such cases it would have been much better to introduce new coefficients that for example could be denoted $k_{cat(\text{-t})}$ and $k_{cat(\text{-t})}/K_{\mathrm{m}}$ where (-t) shows that the coefficient is not based on active site titration.

Detection of the Substrate Specificity of an Enzyme with or without Knowledge of The Absolute Concentration of the Functional Active Sites

Under some conditions it is not possible to determine the concentration of functional enzyme active sites and hence, the absolute value of the kinetic coefficients. This will of course limit our ability to compare the absolute activity of enzymes, but it is still possible to obtain various kinetic characteristics such as the substrate specificity for an enzyme and compare this with the substrate specificity of another enzyme, detection of inhibitory compounds and reaction mechanism. A typical example is our early studies of DADHs [38, 39]. We intended to determine the topology of the enzyme active site long before a 3D-structure of DADH was available. As the topology of the active-site determines the substrate specificity of the enzyme, we decided to investigate the substrate specificity of the enzyme by using approximately 100 different structurally well defined alcohols (primary, secondary, linear, cyclic and bi-cyclic). We faced several problems during these early studies of DADH, one was the small amounts of enzyme available which were not enough to perform active site titration, and hence it was not possible to determine the absolute values of the various kinetic coefficients. The second was the large amount of alcohols that we planned to use, and how to determine the substrate specificity without obtaining all the kinetic coefficients and their absolute values. This of course required optimal reaction conditions in order to determine the specificity of the various DADHs. I will try do describe some of the problems and how we solved them.

Quantitative estimation of functional enzyme without active site titration

How did we ensure that the same amount of functional enzyme was used in each experiment? This problem was solved simply by using a high saturating concentration of ethanol as a standard at optimal conditions as described below. We also showed that the enzyme activity under the condition used with a fixed ethanol concentration was linear with the variation in enzyme concentration ($v = k$ x [e]), although the absolute [e] was not known. We therefore presented all data as V_m, V_m/K_{m} and activity at fixed alcohol concentrations (see below) relative to the activity of ethanol [32, 38, 39]. Later on, when we had enough

DADH to perform active-site titrations, we used the standard conditions above in the development of a rate assay that was calibrated against the titration [31, 32]. This of course allowed us to convert all old relative data to absolute data.

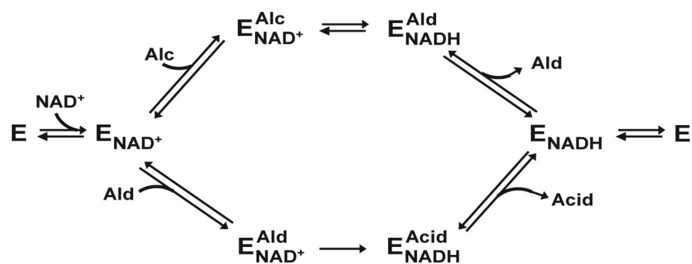
Detection of substrate specificity using a single coenzyme concentration

Which of the kinetic coefficients reflect an enzyme's substrate specificity? As DADH is a two substrate enzyme as described above, the substrate specificity is reflected in the kinetic coefficient N_2 in Equation 3 or K_{m2}/k_{cat} in Equation 5 for various alcohols. Our aim was to get a picture of the substrate specificity by using a fixed NAD^+ concentration, vary the concentration of some selected alcohols and thereafter determine the activity for all alcohols at a fixed concentration. In order to use a fixed NAD^+ concentration, it is important that this is high enough so the obtained (app)k_{cat}/K_{m2} and (app)k_{cat} values are as close as possible to the values for an infinite coenzyme concentration. This requires that $K_{m1}/(k_{cat}$ $[NAD^+])$ is much less than $1/k_{cat}$ and that $K_{m2}\ K_{ia}/(k_{cat}\ [NAD^+])$ is much less than K_{m2}/k_{cat}. The problem was to find the experimental conditions that were optimal in order to obtain reliable results. We decided to use a temperature that was used in the classical experiments on horse liver ADH by Theorell and McKinley-McKee [40] and by Dalziel [41]. Our initial experiments revealed that optimal conditions were obtained using 0.1 M glycine–NaOH buffer pH 9.5 and a fixed concentration of 0.5 mM of NAD^+. The reason to choose such a high pH compared to physiological pH is of course the equilibrium of the reaction and the amount of NAD^+ needed to obtain acceptable values of (app)k_{cat} and (app)k_{cat}/K_{m2}. As an example, at neutral pH with a NAD^+ concentration of 1 mM, $N_1/[NAD^+]$ and $N_{12}/[NAD^+]$ are approximately the same as N_0 and N_2, respectively [42, 43]. Using 10 mM of NAD^+ would have reduced the ratios to be 5 – 10% of the corresponding N coefficient. However at basic pH (9.5 – 10) these two relations are approximately 2% of corresponding N_0 and N_2 coefficient, using 0.5 mM of NAD^+ [42, 43]. These calculations are based on the two substrates ethanol and propan-2-ol using the *D. melanogaster* alleloenzyme ADH^S and the *D. lebanonensis* ADH [42, 43]. This can be compared with results for sheep liver Sorbitol dehydrogenase (SDH), where the corresponding relations $(1+ N_1/(N_0\ [NAD^+])$ and $1 + N_{12}/(N_2\ [NAD^+]))$ using 1 mM NAD^+ are close to 2 at both pH 7.4 and 9.5 using sorbitol as varied substrate [34]. Studies of this SDH revealed that the substrate specificity was the same at neutral and at basic pH [44].

DADH is also able to oxidize aldehydes in the presence of NAD^+ to their corresponding acids (Equation 2) [3, 4, 45, 46]. At pH 7.0, it is not possible to follow this reaction by determining the production of NADH. This is due to the dismutation reaction (Equation 6; which is the sum of Equations 1 and 2), i. e. as fast as NADH is produced, it reacts with the aldehyde and produces alcohol.

$$2\ \text{aldehyde} + H_2O \leftrightarrow \text{carboxylate}^- + \text{alcohol} + H^+ \qquad (6)$$

However, above pH 9.0 it has been possible to detect NADH production with DADH as the reduction reaction of aldehyde to alcohol is slower than at neutral pH [3, 4, 45, 46], and hence an unequal amount of alcohol and acid is produced in the dismutation reaction. It has been argued that the increase in A_{340nm}, i.e. the release of NADH, is not a direct measure of the aldehyde oxidation reaction and acid production, and that the resulting kinetic values cannot be compared with those for alcohol dehydrogenation. This indicates that aldehyde oxidation can only be studied with methods such as ^{1}H-NMR, gas chromatography or pH-stat titrations. Due to the amount of enzyme needed, as well as initial-rate measurements cannot be performed with the two former methods, one would expect that this would limit the possibility of doing kinetic studies on the aldehyde oxidation reaction. Even if this is correct to a certain extent, we have shown that it is possible to do kinetic studies by following the initial-rate production of NADH at pH 9.5, by using a very sensitive filter fluorimeter specially built to study dehydrogenase reactions [4]. With this instrument we could detect the continuous production of NADH, with a detection limit as low as 10 nM. We performed substrate specificity studies, as well as detecting kinetic coefficients for the aldehyde oxidation reaction and compared this with both the alcohol oxidation reaction and aldehyde reduction reaction [4]. The combination of dead-end and product inhibitors was used to determine the reaction mechanism for the aldehyde oxidation pathway, which like the interconversion between alcohols and aldehydes was consistent with a compulsory ordered mechanism as shown in Scheme 1. It is important to emphasize that it is necessary to avoid buffers containing primary or secondary amine groups, as these formed Schiff bases with the aldehydes. This shows the possibilities to do studies of enzymes if optimal reaction conditions and optimal instrumentation is used, and that some type of studies is not possible to perform at neutral pH.



Scheme 1. Reaction mechanism for DADH. The upper pathway shows the interconversion between an alcohol (Alc) and an aldehyde (Ald), and the lower pathway the oxidation of an aldehyde (Ald) to a carboxylic acid (Acid). The mechanism for these reactions was consistent with a compulsory ordered pathway, where the coenzymes form binary enzyme complexes.

Determination of substrate specificity using a single fixed alcohol and NAD$^+$ concentration

In order to use only one alcohol concentration, which is the optimal concentration to use? As N_2 (K_{m2}/k_{cat}) reflects the activity at low alcohol concentrations, one should use a concentration that is below K_m. We used 1 mM of the different alcohols, which was assumed to be an acceptable concentration. This also appeared to be the case for the primary alcohols and a lot of the secondary alcohols. Although this concentration proved to be a little too high with respect to some of the secondary alcohols, it reflected in an acceptable way the (app)k_{cat}/K_{m2} values in those cases where these were obtained [32, 38, 39]. The substrate specificity obtained at pH 9.5 has been shown to reflect the substrate specificity at neutral pH for DADH [42, 43].

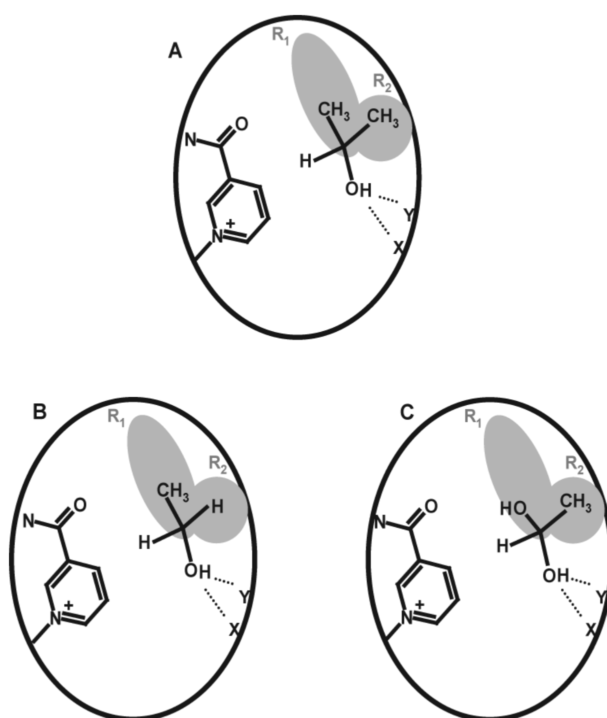


Figure 2. Schematic representation of the alcohol binding site in ternary DADH-NAD$^+$-substrate complexes. The binding of (**A**) propan-2-ol, (**B**) ethanol and (**C**) acetaldehyde (diol) is shown. The hydrophobic and bifurcated part of the enzyme active site that interacts with the alkyl groups in alcohols and aldehydes is shown in grey and labelled as R_1 and R_2. Also shown is the nicotinamide part of the oxidized coenzyme NAD$^+$ and the OH-group of the substrates that interacts with the OH-group in the two conserved residues tyrosine-151 (Y) and serine-138 (X) using *D. lebanonensis* numbering.

Professor Ladenstein and his group at Karolinska Institutet in Sweden obtained the 3D-structure of several ternary DADH-NAD^+-ketone complexes through X-ray crystallography [10], and their description of the topology of the active site was exactly as we depicted from our substrate specificity studies more then 15 years earlier [32, 38, 39]. The kinetic and X-ray crystallography data showed a hydrophobic, bifurcated substrate-binding site in DADH, which results in optimal binding and activity with secondary alcohols (Fig. 2a). Kinetic and X-ray crystallographic data has also shown that the alkyl chain in ethanol and other primary alcohols as well as aldehydes during reduction with NADH to alcohols binds to the R_1 part of this bifurcated alcohol binding part of the active site (Fig. 2b) [10, 47]. However, in the oxidation of aldehydes to acids, the alkyl chains in the aldehyde binds to the R_2 binding part of the active site (Fig. 2c) [11].

Additives in a Purified Enzyme Preparation May Alter the Biochemical Properties of the Enzyme

In this part I will take up the importance of a careful description of an enzyme assay, i.e. the conditions used including the concentrations of all the constituents in the assay. The example used shows that the amount of additives present in a preparation of proMMP-2 determines whether or not trypsin will act as an activator of this MMP.

The literature states that serine proteinases like trypsin cannot activate proMMP-2. This has been based on very careful studies by Okada *et al.*, [48] in which they studied the activation of proMMP-2 by the organic mercury compound 4-aminophenyl mercury acetate (APMA). In this study, several proteinases including trypsin were also tested as proMMP-2 activators, and none of these activated the enzyme. The conditions used for the activation with APMA are very well documented, while the conditions used when trypsin and the other proteinases were tested, are less well documented. They used various amounts of trypsin (0.1 – 100 µg/ml) at 22 °C from 5 minutes to 30 hours. What was not explicitly cited was the concentration used of $CaCl_2$, and if they used Brij-35 in the assay and if so, what was the concentration. In another article, it was shown that trypsin-2 is an activator of proMMP-9, but could only partly activate proMMP-2 [49]. However, nothing was mentioned with respect to reaction conditions such as added Brij-35 or $CaCl_2$.

These results fitted badly with my own studies on the expression of MMP-2 from cultured fibroblasts [50,51]. After harvesting the cell-conditioned serum-free medium, we used to add $CaCl_2$, BSA and Hepes (pH 7.5) to a final concentration of 10 mM, 0.2 % and 0.1 M respectively. This was done in order to protect the enzyme in the freezing (−20 °C) and thawing processes. The proMMP-2 in these serum-free media was always activated by trypsin, and we showed that this was not due to the activation of another MMP (collagenase 1/MMP-1) in the media, that then could activate proMMP-2 [51]. In a recent study, we did check whether trypsin could activate recombinant proMMP-2 [35]. In these studies we decided to test whether the discrepancies between our results, using cell conditioned media and those that used purified proMMP-2, could be ascribed to differences in experimental

reaction conditions, or, that the activation of proMMP-2 in the cell conditioned media actually was due to trypsin-induced activation of a latent MMP-2 activator in the media and not through a direct activation of proMMP-2.

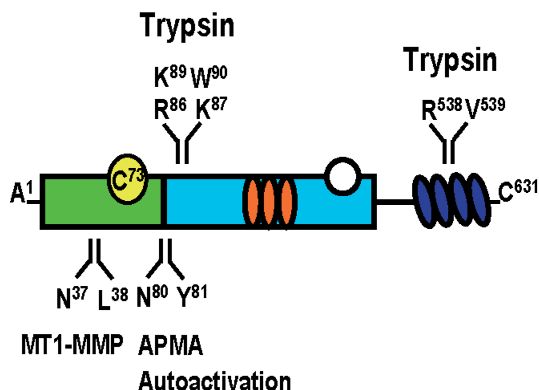


Figure 3. Schematic drawing showing the cleavage sites in proMMP-2 produced by MT1-MMP, APMA, autoactivation and trypsin. MT1-MMP cleaves N-terminal for the invariant C^{73} that is linked to the active site zinc in the proenzyme. The intermediate formed is further processed by autoactivation that generates the fully active 62 kDa form of MMP-2. Treatment of proMMP-2 with AMPA results in autoactivation. Trypsin cleaves C-terminal for the autoactivation site, and at several sites in the C-terminal region, ending up with a cleavage between R^{538} and V^{539} generating an active 50 kDa form.

The commercial recombinant proMMP-2 used in our studies was delivered from Chemicon, and contained 100 µg/ml proMMP-2 in 5 mM Tris–HCl (pH 7.5), 0.1 mM $CaCl_2$ and 0.005 % Brij-35. In our activation experiments with trypsin, this proMMP-2 stock solution always ended up 30 – 40 times diluted in 0.1 M Hepes, pH 7.5 prior to the addition of the different amounts of trypsin, $CaCl_2$ and Brij-35. Other conditions varied were temperature (4, 16, 22 and 37 °C) and incubation time with trypsin (2 minutes to 24 hours). Our results showed that trypsin is actually an activator of proMMP-2 that first removes the pro-domain from the 72 kDa proMMP-2 and generates an active 62 kDa form. This is followed by a trypsin-induced successive removal of the most C-terminal parts of the hemopexin-like domain that ends up in a 50 kDa active form of the enzyme as shown in Fig. 3. Without exogenous added $CaCl_2$ and Brij-35, trypsin induced activation at the low temperatures, while at 37 °C, the proMMP-2 was only degraded. Both $CaCl_2$ and Brij-35 stabilized the MMP, which could be activated at 37 °C if only one of these compounds were present. However in the presence of 0.05 % Brij-35, trypsin-induced activation decreased with increasing concentrations of $CaCl_2$. At 5 and 10 mM $CaCl_2$ (approximately 5 – 10 times the physiological concentration in tissues) only a small fraction was activated and almost all the enzyme remained in the proform. Thus the discrepancy in the literature cannot be ascribed experimental faults or the activation of an unknown proMMP-2 activator in cell

conditioned media, but was due to various reaction conditions. The trypsin-induced activation of proMMP-2 generates an active MMP-2 with a slightly shorter N-terminal than the enzyme activated by the assumed most important biological activator, MT1-MMP, or the organic mercurial compound APMA (Fig. 3). This difference in structure also resulted in an altered capacity of the enzyme to degrade the biological substrate, gelatin, and a chromogenic substrate, as well as an altered binding strength (K_i) to the biological inhibitor TIMP-1 [35].

These results clearly demonstrate the importance of various additives and to report their concentration, as they may affect the parameters studied. By reporting all the additives and their concentrations, authors allow others to extend their investigation as well as to test the substance in the published results. As shown above, due to the presence of various additives in the reaction assay, an erroneous statement about a biological parameter of an enzyme has been introduced in the literature which is hard to erase.

Conclusions

In order to obtain a full description of the kinetic coefficients, the concentration of functional enzyme active sites is required. This should be obtained by a method based on active-site titration.

If it is not possible to obtain the amount of functional enzyme by active site titration methods, my view is that it is wrong to present kinetic coefficients like k_{cat} and k_{cat}/K_m using units such as s^{-1} and mM^{-1} s^{-1}, respectively. In a lot of cases it is much better to present the kinetic coefficients as specific activities or relative activities using V_m and V_m/K_m. In other cases it may be better to introduce new kinetic coefficients, which for example could be denoted $k_{cat(-t)}$ and $k_{cat(-t)}/K_m$ (using units such s^{-1} and $mM^{-1}s^{-1}$), where (–t) shows that the catalytic activity is not based on active site titration.

It should not be necessary to stress that a clear description of conditions used, including all additives, should be reported.

Standardization of parameters such as pH and temperature can be done to a certain extent where it is appropriate.

References

[1] Boyce, S., Tipton, K., McDonald, A.G. (2003) Extending enzyme classification with metabolic and kinetic data: Some difficulties to be resolved. In: *1st International Beilstein Workshop on Experimental Standard Conditions of Enzyme Characterizations* (Hicks, M., Kettner, C., Eds), pp. 17–43, Beilstein-Institut, Rüdesheim/Rhein, Germany.

[2] Winberg, J.O., McKinley-McKee, J.S. (1992) Kinetic interpretations of active site topologies and residue exchanges in Drosophila alcohol dehydrogenases. *Int. J. Biochem.* **24**:169–181.

[3] Henehan, G.T., Chang, S.H., Oppenheimer, N.J. (1995) Aldehyde dehydrogenase activity of Drosophila melanogaster alcohol dehydrogenase: burst kinetics at high pH and aldehyde dismutase activity at physiological pH. *Biochemistry* **34**:12294–12301.

[4] Winberg, J.O., McKinley-McKee, J.S. (1998) Drosophila melanogaster alcohol dehydrogenase: mechanism of aldehyde oxidation and dismutation. *Biochem. J.* **329**:561–570.

[5] Chambers, G.K. (1991) Gene expression, adaptation and evolution in higher organisms. Evidence from studies of Drosophila alcohol dehydrogenases. *Comp. Biochem. Physiol. [B]* **99**:723–730.

[6] Jörnvall, H., Persson, B., Krook, M., Atrian, S., Gonzalez-Duarte, R., Jeffery, J., Ghosh, D. (1995) Short-chain dehydrogenases/reductases (SDR). *Biochemistry* **34**:6003–6013.

[7] Jörnvall, H., Persson, M., Jeffery, J. (1981) Alcohol and polyol dehydrogenases are both divided into two protein types, and structural properties cross-relate the different enzyme activities within each type. *Proc. Natl Acad. Sci. U S A* **78**:4226–4230.

[8] Kallberg, Y., Oppermann, U., Jörnvall, H., Persson, B. (2002) Short-chain dehydrogenases/reductases (SDRs): Coenzyme-based functional assignments in completed genomes. *Eur. J. Biochem.* **269**:4409–4417.

[9] Benach, J., Atrian, S., Gonzalez-Duarte, R., Ladenstein, R. (1998) The refined crystal structure of Drosophila lebanonensis alcohol dehydrogenase at 1.9 A resolution. *J. Mol. Biol.* **282**:383–399.

[10] Benach, J., Atrian, S., Gonzalez-Duarte, R., Ladenstein, R. (1999) The catalytic reaction and inhibition mechanism of Drosophila alcohol dehydrogenase: observation of an enzyme-bound NAD-ketone adduct at 1.4 A resolution by X-ray crystallography. *J. Mol. Biol.* **289**:335–355.

[11] Benach, J., Winberg, J.O., Svendsen, J.S., Atrian, S., Gonzalez-Duarte, R., Ladenstein, R. (2005) Drosophila alcohol dehydrogenase: acetate-enzyme interactions and novel insights into the effects of electrostatics on catalysis. *J. Mol. Biol.* **345**:579–598.

[12] Geer, B.W., Heinstra, P.W. H., Kapoun, A.M., Van der Zel, A. (1990) Alcohol dehydrogenase and alcohol tolerance in drosophila melanogaster. In: *Ecological and Evolutionary Genetics of Drosophila* (Barker, J.S. F. e. a., Ed.), Plenum Press, New York.

[13] Allemann, R.K., Hung, R., Benner, S.A. (1988) Stereochemical profile of the dehydrogenases of drosophila melanogaster. *J. Am. Chem. Soc.* **110**:5555–5560.

[14] Benner, S.A., Nambiar, K.P., Chambers, G.K. (1985) A stereochemical imperative in dehydrogenases: New data and criteria for evaluating function-based theories in bioorganic chemistry. *J. Am. Chem. Soc.* **107**:5513–5517.

[15] Winberg, J.O., Brendskag, M.K., Sylte, I., Lindstad, R.I., McKinley-McKee, J.S. (1999) The catalytic triad in drosophila alcohol dehydrogenase: pH, temperature and molecular modelling studies. *J. Mol. Biol.* **294**:601–605.

[16] Winberg, J.O., McKinley-McKee, J.S. (1988) Drosophila melanogaster alcohol dehydrogenase. Biochemical properties of the NAD+-plus-acetone-induced isoenzyme conversion. *Biochem. J.* **251**:223–227.

[17] Winberg, J.O., McKinley-McKee, J.S. (1994) Drosophila melanogaster alcohol dehydrogenase: product-inhibition studies. *Biochem. J.* **301**:901–909.

[18] Winberg, J.O., Thatcher, D.R., McKinley-McKee, J.S. (1982) Alcohol dehydrogenase from the fruitfly Drosophila melanogaster. Inhibition studies of the alleloenzymes AdhS and AdhUF. *Biochim. Biophys. Acta* **704**:17–25.

[19] Winberg, J.O., Thatcher, D.R., McKinley-McKee, J.S. (1983) Drosophila melanogaster alcohol dehydrogenase: an electrophoretic study of the AdhS, AdhF, and AdhUF alleloenzymes. *Biochem. Genet.* **21**:63–80.

[20] Nagase, H., Woessner, J.F., Jr. (1999) Matrix metalloproteinases. *J. Biol. Chem.* **274**:21491–21494.

[21] Rawlings, N.D., Tolle, D.P., Barrett, A.J. (2004) MEROPS: the peptidase database. *Nucleic Acids Res.* **32**:D160–D164.

[22] Overall, C.M., López-Otin, C. (2002) Strategies for MMP inhibition in cancer: Innovations for the post-trial era. *Nature Rev. Cancer* **2**:657–672.

[23] Vihinen, P., Kähäri, V.M. (2002) Matrix metalloproteinases in cancer: Prognostic markers and therapeutic targets. *Int. J. Cancer* **99**:157–166.

[24] Nagase, H. (1997) Activation mechanisms of matrix metalloproteinases. *Biol. Chem.* **378**:151–160.

[25] Brew, K., Dinakarpandian, D., Nagase, H. (2000) Tissue inhibitors of metalloproteinases: evolution, structure and function. *Biochim. Biophys. Acta* **1477**:267–283.

[26] Dalziel, K. (1957) Initial steady state velocities in the evaluation on enzyme-coenzyme-substrate reactions. *Acta Chem. Scand.* **11**:1706–1723.

[27] Cleland, W.W. (1977) Determining the chemical mechanisms of enzyme-catalyzed reactions by kinetic studies. *Adv. Enzymol. Relat. Areas Mol. Biol.* **45**:273–387.

[28] Brocklehurst, K. (1996) Active site titration. In: *Enzymology: LabFax* (Engel, P.C., Ed.), pp. 59–66, Academic Press, San Diego.

[29] Theorell, H., Yonetani, T. (1963) Liver alcohol dehydrogenase-DPN-Pyrazole complex: A model of a ternary intermediate in the enzyme reaction. *Biochem. Z.* **338**:537–553.

[30] Ganzhorn, A.J., Green, D.W., Hershey, A.D., Gould, R.M., Plapp, B.V. (1987) Kinetic characterization of yeast alcohol dehydrogenases: Amino acid residue 294 and substrate specificity. *J. Biol. Chem.* **262**:3754–3761.

[31] Winberg, J.O., Hovik, R., McKinley-McKee, J.S. (1985) The alcohol dehydrogenase alleloenzymes AdhS and AdhF from the fruitfly Drosophila melanogaster: an enzymatic rate assay to determine the active-site concentration. *Biochem. Genet.* **23**:205–216.

[32] Winberg, J.O., Hovik, R., McKinley-McKee, J.S., Juan, E., Gonzalez-Duarte, R. (1986) Biochemical properties of alcohol dehydrogenase from Drosophila lebanonensis. *Biochem. J.* **235**:481–490.

[33] Ganzhorn, A.J., Plapp, B.V. (1988) Carboxyl groups near the active site zinc contribute to the catalysis in yeast alcohol dehydrogenase. *J. Biol. Chem.* **263**:5446–5454.

[34] Lindstad, R.I., Hermansen, L.F., McKinley-McKee, J.S. (1992) The kinetic mechanism of sheep liver sorbitol dehydrogenase. *Eur. J. Biochem.* **210**:641–647.

[35] Lindstad, R.I., Sylte, I., Mikalsen, S.O., Seglen, P.O., Berg, E., Winberg, J.O. (2005) Pancreatic trypsin activates human promatrix metalloproteinase-2. *J. Mol. Biol.* **350**:682–698.

[36] Minond, D., Lauer-Fields, J.J., Nagase, H., Fields, G.B. (2004) Matrix metalloproteinase triple-helical peptidase activities are differentially regulated by substrate stability. *Biochemistry* **43**:11474–11481.

[37] Park, H.I., Turk, B.E., Gerkema, F.E., Cantley, L.C. (2002) Peptide substrate specificities and protein cleavage sites of human endometastase/matrilysin-2/matrix metalloproteinase-26. *J. Biol. Chem.* **38**:35168–35175.

[38] Hovik, R., Winberg, J.O., McKinley-McKee, J.S. (1984) Drosophila melanogaster alcohol dehydrogenase: substrate specificity of the ADHF alleloenzyme. *Insect Biochem.* **14**:345–351.

[39] Winberg, J.O., Thatcher, D.R., McKinley-McKee, J.S. (1982) Alcohol dehydrogenase from the fruitfly Drosophila melanogaster. Substrate specificity of the alleloenzymes AdhS and AdhUF. *Biochim. Biophys. Acta* **704**:7–16.

[40] Theorell, H., McKinley-McKee, J.S. (1961) Liver alcohol dehydrogenase: I. Kinetics and equilibria without inhibitors. *Acta Chem. Scand.* **15**:1797–1810.

[41] Dalziel, K. (1962) Kinetic studies of liver alcohol dehydrogenases. *Biochem. J.* **84**:244–254.

[42] Brendskag, M.K., McKinley-McKee, J.S., Winberg, J.O. (1999) Drosophila lebanonensis alcohol dehydrogenase: pH dependence of the kinetic coefficients. *Biochim. Biophys. Acta* **1431**:74–86.

[43] Winberg, J.O., McKinley-McKee, J.S. (1988) The AdhS alleloenzyme of alcohol dehydrogenase from Drosophila melanogaster. Variation of kinetic parameters with pH. *Biochem. J.* **255**:589–599.

[44] Lindstad, R.I., Köll, P., McKinley-McKee, J.S. (1998) Substrate specificity of sheep liver sorbitol dehydrogenase. *Biochem. J.* **330**:479–487.

[45] Heinstra, P.W., Geer, B.W., Seykens, D., Langevin, M. (1989) The metabolism of ethanol-derived acetaldehyde by alcohol dehydrogenase (EC 1.1.1.1) and aldehyde dehydrogenase (EC 1.2.1.3) in Drosophila melanogaster larvae. *Biochem. J.* **259**:791–797.

[46] Moxon, L.N., Holmes, R.S., Parsons, P.A., Irving, M.G., Doddrell, D.M. (1985) Purification and molecular properties of alcohol dehydrogenase from drosophila melanogaster: Evidence from NMR and kinetic studies for function as an aldehyde dehydrogenase. *Comp. Biochem. Physiol. [B]* **80B**:525–535.

[47] Winberg, J.O., Martinoni, B., Roten, C., McKinley-McKee, J.S. (1993) Drosophila alcohol dehydrogenase: stereoselective hydrogen transfer from ethanol. *Biochem. Mol. Biol. Int.* **31**:651–658.

[48] Okada, Y., Morodomi, T., Enghild, J.J., Suzuki, K., Yasui, A., Nakanishi, I., Salvesen, G., Nagase, H. (1990) Matrix metalloproteinase 2 from human rheumatoid synovial fibroblasts. Purification and activation of the precursor and enzymic properties. *Eur. J. Biochem.* **194**:721–730.

[49] Sorsa, T., Salo, T., Koivunen, E., Tyynela, J., Konttinen, Y.T., Bergmann, U., Tuuttila, A., Niemi, E., Teronen, O., Heikkila, P., Tschesche, H., Leinonen, J., Osman, S., Stenman, U.H. (1997) Activation of type IV procollagenases by human tumor-associated trypsin- 2. *J. Biol. Chem.* **272**:21067–21074.

[50] Winberg, J.O., Gedde-Dahl, T. (1986) Gelatinase expression in generalized epidermolysis bullosa simplex fibroblasts. *J. Invest. Dermatol.* **87**:326–329.

[51] Winberg, J.O., Gedde-Dahl, T., Jr. (1992) Epidermolysis bullosa simplex: expression of gelatinase activity in cultured human skin fibroblasts. *Biochem. Genet.* **30**:401–420.