MINI REVIEW

MARIA JEŻEWSKA

DIVERGENCY OF STRUCTURE AND FUNCTION OF VERTEBRATE XANTHINE: NAD$^+$ OXIDOREDUCTASE *

Institute of Biochemistry and Biophysics, Polish Academy of Sciences
Rakowiecka 36, 02-532 Warsaw, Poland

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Selection pressure for increasing metabolic flux through a define metabolic pathway affects the enzyme levels, enzyme structure and their kinetic properties. These aspects exemplified by xanthine oxidoreductase from vertebrates of various type of nitrogen excretion are discussed. Two trends in evolutionary kinetic changes of oxypurine hydroxylyating activity could be distinguished. Changes in the subunit structure and kinetic properties suggest that the domain catalysing oxypurine hydroxylation and the one cooperating with NAD$^+$ evolved through separate pathways.

Natural selection during phylogenesis may exert probably several effects on enzymic catalysis, changing the enzyme levels, structure and enzyme kinetic properties. It seems that the enzyme of purine metabolism in vertebrates are of first choice for following phylogenetic trends, because of diversified intensity of metabolite flux through this pathway during phylogenetic development.

In the whole animal kingdom (except in parasitic organisms) purine nucleotide biosynthesis de novo provides substrates for biosynthesis of nucleic acids and a variety of compounds taking part in many metabolic reactions. The nitrogen of uric acid — the final purine compound in the purine catabolic pathway — accounts for only a few percent of the total nitrogen excreted, the rest being nitrogen of the end-products of protein metabolism: ammonium and urea in animals, ammonio- or ureotelic, according to the prevalence of one of these compounds in excreta. In uricotelic animals, the purine nucleotide biosynthesis de novo and catabolic pathway serve also for elimination of superfluous nitrogen of protein metabolism, and in this case the uric acid nitrogen represents over 90% of the total nitrogen excreted. The type of

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nitrogen excretion is not related to the systematic class to which animal belongs: teleostean fishes share ammonotelism with some amphibians; other fishes and amphibians and all mammals are ureotelic. Some reptiles are ammamo- or ureotelic, while some other are uricotelic. All birds are uricotelic. Moreover, in many lower vertebrates a transition from ammonotelism to ureotelism and vice versa is observed under varying life conditions [1].

Comparative studies on the enzyme activities, substrate and product concentrations in liver — the main site of uric acid synthesis — have evidenced many differences between the ureotelic rat and uricotelic chick [2, 3]. Moreover the metabolic flux through the purine metabolic pathway (Scheme 1) is 15-20 times more intensive in chicken. In chicken liver the bulk of IMP synthesized de novo is directly degraded to hypoxanthine and next to xanthine and uric acid. In contrast, in rat liver IMP is preferably transformed into adenine and guanine nucleotides. In this case, hypoxanthine derived from adenine nucleotide is to a great extent utilized for IMP resynthesis and only in part it is hydroxylated to xanthine which, together with xanthine derived from guanine nucleotides, is hydroxylated to uric acid.

In all organisms the last two irreversible reactions: hypoxanthine→xanthine→uric acid are catalyzed by the NAD⁺-dependent xanthine oxidoreductases. The differences in purine metabolism presented in Scheme 1 suggest that these enzymes have opposite tasks in ureo- and uricotelic

Scheme 1. Metabolite flux intensity through the purine metabolic pathway in liver of rat, I; and chicken, II

\[ \text{NH}_4^+ \]
\[ \text{gln} \]
\[ \text{IMP} \]
\[ \text{G} \]
\[ \text{urea} \]
\[ \text{Ado} \]
\[ \text{Ino} \]
\[ \text{Hyp} \]
\[ \text{Xan} \]
\[ \text{Uri} \]
\[ \text{Gua} \]
\[ \text{Guo} \]
organisms: in the latter they provide for efficient elimination of excess hypoxanthine, in the former, they spare hypoxanthine for IMP resynthesis. The question arises how this problem is resolved?

Changes in the enzyme level

Discussing variations in nitrogen excretion Wilson et al. [4] suggested that the major differences in the nitrogen excretory metabolism among vertebrates could be mainly due to the differences in relative activities of the enzymes involved. Consistently with Scheme 1, xanthine oxidoreductase activity is higher in liver of adult uricotelic vertebrates than of ammono- and ureotelic ones (Table 1); however, it is only 2.5-fold higher in hen than in rat, whereas a 20-fold difference occur in the metabolite flux through the purine nucleotide pathway in these animals. Two fold difference between liver of toad and rat (both ureotelic) in the enzyme activity suggests that the enzyme level is related to the intensity of overall metabolism rather than to the nitrogen excretion type. This suggestion is supported by a severalfold difference in xanthine oxidoreductase activity found in liver of toad during the winter sleep and active

Table 1

Xanthine: NAD⁺ oxidoreductase activity in liver of various vertebrates

<table>
<thead>
<tr>
<th>Animal</th>
<th>Xanthine oxidoreductase activity (nmol/min per g of fresh tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonotelic</td>
<td></td>
</tr>
<tr>
<td>Fish <em>Cyprinus carpio</em> (active life)</td>
<td>24</td>
</tr>
<tr>
<td>Ureotelic</td>
<td></td>
</tr>
<tr>
<td>Toad <em>Bufo viridis</em> (hibernating) (active life)</td>
<td>12</td>
</tr>
<tr>
<td>* Rat Wistar</td>
<td></td>
</tr>
<tr>
<td>(adult)</td>
<td>125</td>
</tr>
<tr>
<td>(14th day after birth)*</td>
<td>40</td>
</tr>
<tr>
<td>(day of birth)</td>
<td>150</td>
</tr>
<tr>
<td>(fetus)</td>
<td>50</td>
</tr>
<tr>
<td>Uricotelic</td>
<td></td>
</tr>
<tr>
<td>Grass snake <em>Natrix natrix</em> (hibernating)</td>
<td>360</td>
</tr>
<tr>
<td>Hen <em>Gallus gallus</em></td>
<td>300—400</td>
</tr>
<tr>
<td>** Chick</td>
<td></td>
</tr>
<tr>
<td>Chick embryo (2 days before hatching)</td>
<td>5</td>
</tr>
</tbody>
</table>

* after Hashimoto [5]; * — critical day of switching metabolism from neonatal to infantile life.
** after DeLapp & Fisher [6]. Data for fish and snake [7, 8, respectively], for toad, hen and chick embryo [9, 10a, b, respectively].
life periods. In turn, in liver of rat fetus the enzyme activity [5] is higher than in chick embryo liver [11]. In both animal species this activity increases further during growth (Table 1). Only in adult chick and rat the level of xanthine oxidoreductase activity responds in a clearly different manner to many factors and varying life conditions [12, 13, 14]. Thompson et al. [13] have found that changes in the rate of the enzyme synthesis but not its degradation, are responsible for these activity changes in chick liver. It seems that such a control on the level of gene expression, which takes several hours or even days, determines long-term adaptive response to varying conditions of animal life rather than it regulates the opposite functions of this enzyme in urico- and ureotelmism.

Changes in kinetic properties

In 1970 Della Corte & Stirpe [15] have postulated that differences in kinetic properties found between rat and chicken xanthine oxidoreductase may reflect the enzyme adaptation to a different intensity of purine metabolism in these animals. Recently, Petterssen [16] considered from a theoretical point of view in which way the evolutionary pressure for increasing metabolic flux could affect kinetic parameters of the enzyme. He has concluded that such selective pressure should tend to increase the catalytic constant (k_{cat}) of the enzyme, whereas the Michaelis constant (K_m) may either decrease or increase. Studies on the xanthine oxidoreductases from liver of several vertebrates: rat [17, 18, 19], snake and carp [7, 8, respectively], turkey (Kamiński & Jeżewska, unpublished), toad [9], hen and chick embryos [10a, b] evidenced a great divergency in kinetic properties, corresponding with various in vivo physiological conditions. Also, these studies pointed to the unique properties of mammalian enzyme as compared to the other vertebrate xanthine oxidoreductase (Table 2) [20].

The K_m value for NAD^+ tend to decrease in xanthine oxidoreductase from uricotelic animals. This may be considered as a result of selection for efficient competition for NAD^+ with the other NAD^+-dependent enzymes under conditions of increasing importance of the purine catabolic pathway. In turn, differences in K_m values for oxypurines suggest the existence of two opposite trends in the positive selection: one — to increase K_m for hypoxanthine and the K_{mHyp}/K_{mXan} ratio associated with enhanced metabolic flux of purine compounds (uricotelism), the second — to decrease K_m for hypoxanthine and the K_{mHyp}/K_{mXan} ratio (in ureotelic mammals). A similar tendency is evident when the enzyme-saturating concentrations of hypoxanthine are compared. Moreover, the ratio of saturating concentrations ([Hyp]/[Xan]) as well as the ratio of hydroxylation rates (V_{Hyp}/V_{Xan}) are increased for xanthine oxidoreductases of uricotelic animals. It is interesting that the chick embryo xanthine oxidoreductase, in respect of kinetic parameters, resembles the toad rather than the hen enzyme (Table 2).
Table 2
Some kinetic parameters of xanthine: NAD⁺ oxidoreductases from vertebrates
Data after [7, 8, 9, 10a, b, 18]

<table>
<thead>
<tr>
<th>Animal</th>
<th>Apparent $K_{m}$ (μM) for NAD⁺</th>
<th>Enzyme-saturating concentrations (μM) of Hyp</th>
<th>Enzyme-saturating concentrations (μM) of Xan</th>
<th>Ratio of hydroxylation rates $V_{Hyp}/V_{Xan}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat (Wistar)</td>
<td>22.4 2.4 1.25</td>
<td>20 20</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>Fish (Cyprinus carpio)</td>
<td>21.5 4.5</td>
<td>40 40</td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td>Toad (Bufo viridis)</td>
<td>27.0 7.0 12.0</td>
<td>60 50</td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td>Snake (Natrix natrix)</td>
<td>14.4 12.3 14.7</td>
<td>100 50</td>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td>Hen (Gallus gallus)</td>
<td>3.1 12.0 33.0</td>
<td>200 100</td>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td>Chick embryo</td>
<td>2.8 4.5 7.0</td>
<td>100 100</td>
<td></td>
<td>1.1</td>
</tr>
</tbody>
</table>

* resulting from computer simulation of the Hyp→Xan→Uri hydroxylation [21].

** measured at saturating concentrations of substrates.

Two basic patterns of the time-course of Hyp→Xan→Uri hydroxylation [20] have been found (Figs. 1, 2 and 3); the first is characteristic for xanthine oxidoreductases from toad, snake, hen and chick embryo; the other one — for the rat enzyme (the fish enzyme exhibits mixed properties). The most striking differences involve the relationship between the rate of uric acid formation and the initial hypoxanthine concentration (Fig. 3). In the case of all vertebrates examined except fish and rat, the uric acid formation does not depend on the

Fig. 1. Patterns of hypoxanthine→xanthine→uric acid hydroxylation catalysed by rat and hen xanthine oxidoreductases. Enzyme activity was equal in experiments with rat, —, and hen, --, enzyme: a, hypoxanthine utilization; b, xanthine accumulation; c, uric acid formation.
Fig. 2. Effect of initial hypoxanthine concentration on xanthine accumulation during Hyp→Xan→Uri hydroxylation catalysed by xanthine oxidoreductases. --, Rat enzyme; ---, hen enzyme; numbers on curves denote initial hypoxanthine concentrations (µM) used in experiments.

Fig. 3. Effect of initial hypoxanthine concentration on uric acid formation during Hyp→Xan→Uri hydroxylation catalysed by xanthine oxidoreductases. --, Rat enzyme; ---, hen enzyme; numbers on curves denote initial hypoxanthine concentrations (µM) used in experiments.
initial hypoxanthine concentration. Such a pattern, together with a slow utilization of accumulated xanthine (Figs. 1, 2) allows a partition of the nitrogen waste load between xanthine and uric acid. This may be important for uricotelic animals, because of a more efficient transport of two hardly soluble substances than of only one but in a double quantity. Thus, it can be assumed that the elimination of superfluous hypoxanthine in uricotelic vertebrates becomes possible because of characteristic changes of kinetics parameters of their xanthine oxidoreductase.

The patterns of hypoxanthine hydroxylation by the rat and carp enzymes evidence the inverse relationship between the initial hypoxanthine concentration and uric acid formation (Fig. 3). This indicates a competition between hypoxanthine and accumulating (Fig. 2) xanthine for the enzyme active centre. This could be helpful for saving hypoxanthine for IMP resynthesis (Scheme 1), e.g. under hypoxic conditions when nucleotide catabolism increases and both oxypurines may accumulate. A conspicuous differences in inhibition of xanthine oxidoreductases by NADH (Fig. 4) from

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Fig. 4. Patterns of inhibition of xanthine oxidoreductases by NADH accumulating during xanthine hydroxylation. Catalysis by rat enzyme (a, b) and hen (c, d); —, under conditions of NADH reoxidation (lactate dehydrogenase + pyruvate added to reaction mixture) and ——, under conditions of NADH accumulation. Initial xanthine concentrations used were those saturating the rat enzyme (20 μM) and hen enzyme (120 μM)
rat and other vertebrates [20] offers another possible explanation how the hypoxanthine could be saved in mammals. Only rat xanthine oxidoreductase is significantly and rapidly inhibited by NADH at physiological concentrations [17, 18], thus its activity could be in vivo regulated by changes in the NAD$^+$/NADH ratio. Xanthine oxidoreductases of other vertebrates are only slightly inhibited by NADH at high, and non-physiological concentrations.

The divergency found in the time-course of hypoxanthine hydroxylation most probably results from the differences in the $K_{m\text{Hyp}}/K_{m\text{Xan}}$ and $V_{\text{Hyp}}/V_{\text{Xan}}$ ratios and not from different mechanisms of catalysis of xanthine oxidoreductases from various sources. This is consistent with the results of computer simulation of this process [21]. Simulations for all the enzymes was based on a “ping-pong” mechanism [22] postulated for xanthine oxidoreductases (cf. [23, 24]), and were in good agreement with our experimental data.

Divergency in molecular structure.

Despite the differences in catalytic action, xanthine oxidoreductases seem to be uniform in structure. These enzymes isolated from several sources (cf. [23]) have approximately the same molecular weight (ca 300 000). The enzyme molecule consists of two subunits which have either identical molecular weight

![Scheme 2. Structure and functioning of xanthine oxidoreductase active centre. Various substrates (purines, pteridines, aldehydes and many other compounds) are bound and transformed in the molybdenum site; natural electron acceptors (NAD$^+$ and O$_2$) bind and are reduced at flavin site. Arrows between active centre components represent their cooperation in electron transport. AEA — artificial electron acceptors](image-url)
as in the milk, and chick embryo enzymes [25, 26] or slightly different as in adult chicken enzyme [26]. In each subunit there is an active centre comprising a molybdenum cofactor, one molecule of FAD and four Fe/S groups forming two different clusters of two Fe/S groups each [27]. The sites of the substrate and electron acceptor binding to the active centre of the enzyme and the pathways of electron transport within this centre are given in Scheme 2. The molybdenum cofactor isolated from milk and chicken liver xanthine oxidoreductases has the same structure [28]. The general character of the molecular structure of the enzyme seem to be conservative. However, the protein structure is generally more conserved in evolution than are the amino acid sequences, [29], therefore this uniformity may be only apparent.

By limited proteolysis of xanthine oxidoreductases from milk [30] and liver of rat [31] and chicken [32] it has been proved that the enzyme subunits consist of three distinct globular domains interconnected by short fragments of polypeptide chain (Scheme 3). Both subunits are linked probably by only hydrophobic forces. The molybdenum cofactor together with Fe/S clusters of both types is associated with the largest domain and FAD is embedded in the smaller domain [33]. Differences in the molecular weight of corresponding

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**Scheme 3.** Domains in dimeric xanthine oxidoreductase molecules (after Coughlan [23], changed). A, rat enzyme; B, chicken enzyme; the domain containing FAD is discontinous and consists of two parts. The direction of polypeptide chain from its N-end to the C-end is indicated by arrows. $M_r$ is given below each of the domains.
domains and a lack of homology of the interdomain oligopeptides [23] in mammalian and avian enzyme subunits indicate considerable differences in the amino acid sequences of polypeptide chains. This is also suggested by the differences in the amino acid composition of the milk [33a] and chicken [33b] enzyme (Table 3). Differences in the amino acid sequence lead probably to different arrangement of domains, because some parts of polypeptide chains seem to be displaced from one domain to another one in chick enzyme (Scheme 3). At present one cannot say which structure, that of the milk enzyme or that of chick enzyme, resembles more closely the structure of the ancestral enzyme. In either case, however, the hydrophobic regions binding Mo-cofactor to protein in the domain [34] and responsible for linking two subunits in a dimer (very stable in milk enzyme, less stable in chick enzyme) must obviously be conserved. Also conservative must be these regions in three domains which are engaged in their spatial arrangement to form the active center. However, the known large specificity towards hydroxylated substrates of xanthine oxidoreductases [27, 35, 36] and the differences in position of the –OH group substitution in the substrate molecule [23] suggest structural

<table>
<thead>
<tr>
<th></th>
<th>Amino acid residues (%)</th>
<th>Difference *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Lysine</td>
<td>7.56</td>
<td>8.99</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.76</td>
<td>2.96</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.98</td>
<td>6.76</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.61</td>
<td>—</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>8.33</td>
<td>10.01</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.22</td>
<td>4.40</td>
</tr>
<tr>
<td>Serine</td>
<td>4.89</td>
<td>3.46</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>11.45</td>
<td>9.25</td>
</tr>
<tr>
<td>Proline</td>
<td>4.60</td>
<td>3.96</td>
</tr>
<tr>
<td>Glycine</td>
<td>4.07</td>
<td>5.76</td>
</tr>
<tr>
<td>Alanine</td>
<td>4.64</td>
<td>5.98</td>
</tr>
<tr>
<td>Valine</td>
<td>5.86</td>
<td>7.79</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.26</td>
<td>1.31</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.94</td>
<td>9.49</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.49</td>
<td>9.55</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.64</td>
<td>4.72</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>6.31</td>
<td>4.71</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>2.30</td>
<td>0.39</td>
</tr>
</tbody>
</table>

* Differences in Thr, Ser, Gla, Phe and particularly in half-Cys contents may further increase, when amino acids in a domain (20,000) lacking in xanthine oxidase (Scheme 3) are taken into account.
Divergency in protein environment of Mo-cofactor in these enzymes from various sources. This divergency must be responsible for the differences in kinetic parameters (Table 2) of the enzymes. No data about amino acid residues in the environment of Mo-cofactor are available, except that two lysyl residues, both not-essential for catalytic process, are found in molybdenum domain of the milk xanthine oxidase subunit [37].

The FAD domains in mammalian and avian enzymes differ not only in size (the avian domain is larger and consists of two discontinous parts) but also in their catalytic action. The FAD site is responsible for the reaction with NAD\(^+\), the natural electron acceptor cooperating with all native xanthine oxidoreductases of vertebrates. FAD-cofactor may act through the two redox systems: FAD/FADH\(^-\) and FAD/FADH\(_2\) [38]. All vertebrate xanthine oxidoreductases except that of mammals are stable dehydrogenases acting through the former semiquinone system, stabilized (Scheme 3) by a hypothetical X group [38]. In contrast, mammalian xanthine oxidoreductase occurs in two interconvertible forms: a dehydrogenase form and an oxidase form. Interconversion is accompanied by oxidation or modification of at least one [19] or more [31] —SH groups localized in the third domain, the smallest one (Scheme 3). At the same time the system of FAD/FADH\(^-\) changes to FAD/FADH\(_2\) and a spatial reorientation of flavine ring takes place [39]. Thus, it may be supposed that in mammalian enzyme —SH groups on the terminal fragment of polypeptide chain are required to stabilize the X group endowing the flavin site with dehydrogenase characteristics. On removal of —SH groups, the X group is translocated and the NAD\(^+\) binding site is lost. Because stabilization of X group by —SH groups is not required for dehydrogenase activity of the chick enzyme, one can suppose that X group is of a different nature in mammalian and chick enzyme. So far, two amino-acid residues, tyrosyl and histidyyl, have been identified in the environment of FAD, but none of them was proved to play the role of X group. In chick liver enzyme a tyrosyl residue was postulated to be located in the NAD\(^+\) binding site [40]; such a group has been also suggested to be present in the dehydrogenase form of rat liver enzyme [41]. A histidyyl residue, determining NAD\(^+\)-dependent xanthine hydroxylatation, was found in the environment of FAD in the chick enzyme but not in the milk enzyme [21]. It should be mentioned that transformation of the dehydrogenase form into oxidase form does not change the hydroxylating activity of rat xanthine oxidoreductase [17]. Also the chick enzyme with the blocked NAD\(^+\) binding site exhibits the same xanthine-hydroxylating activity as the native enzyme with the electron acceptors cooperating in the Mo-cofactor and Fe/S sites (Scheme 2) [40]. Therefore, changes in the FAD site have no effect on the catalytic action in the Mo-cofactor site. It would imply therefore that each of the domains may evolve through a separate pathway.
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