STRUCTURE AND EXPRESSION OF XANTHINE DEHYDROGENASE GENES IN THE TRANSLUCENT-SKIN MUTANTS OF THE SILKWORM

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Chapter IV

XDH activity staining on native PAGE gels and cDNA sequencing of the BmXDH genes of the oq mutant

Introduction

Enzyme activity of XDH has been measured in normal silkworms and in translucent-skin mutants (Hayashi, 1960, 1961; Tamura, 1977, 1983). XDH activity was found in the fat body, Malpighian tubules and digestive organs of normal larvae. The three translucent-skin mutant strains og, og1 and oq have been shown to lack XDH activity, which synthesizes uric acid that is accumulated in the skin of normal larvae. However, the previous assay of XDH had disadvantage: it was difficult to exclude background signal completely. Even in the oq mutant larvae that have no uric acid, XDH activity was found to be only five times less than the activity in normal larvae (Tamura, 1983). It was not known whether this low signal in the mutant reflected the actual activity or showed simply the background of the assay system. The previous studies were also unable to distinguish multiple enzymes which contributed to the XDH activity. On the other hand, methods of protein electrophoresis followed by activity staining have been applied to the analysis of XDH and AO (Yen and Glassman, 1965; Prakash et al., 1969; Andres, 1976; Dickinson and Gaughan, 1981; Ori et al., 1997; Sagi et al., 1998; Seo et al., 1998); enzyme activities can be seen as bands that are distinguished from background easily, and multiple enzymes with the same activity can be studied separately. Drosophila XDH was analyzed by native PAGE and activity staining with hypoxanthine as a substrate, and was shown as a single band (Yen and Glassman, 1965). Dickinson et al. (1981) studied
Drosophila AO by native PAGE or agarose gel electrophoresis and staining with benzaldehyde or acetaldehyde as a substrate. They showed that XDH and PO have weak activity to oxidize aldehydes. Arabidopsis thaliana was also shown to have a single band of XDH and three bands of AO (Seo et al., 1998). Two of the AO bands represented two AO genes of Arabidopsis, and the other band is a heterodimer of the two AO subunits (Akaba et al., 1999). Activity staining is a good technique to estimate the relationship between multiple enzymes with the same activity and the corresponding genes.

Cloning of silkworm XDH genes identified two genes, BmXDH1 and BmXDH2. Thus, in this chapter, fat body protein of mutant strains was analyzed by the native PAGE/activity staining of XDH and AO. The major XDH band disappeared in the oq mutant, while the minor band was not lost. This suggested that the oq gene encoded an enzyme that corresponded to the major band of XDH. On the other hand, the og gene seemed to regulate the activity of at least two enzymes. Probably the og protein was involved in the synthesis of MoCo, which regulate the activity of molybdenum oxidoreductases including XDH. The sequences of the BmXDH cDNAs of the oq mutant indicated that the BmXDH1 gene transcript was translated as an incomplete polypeptide that lacked the enzyme activity.
Materials and Methods

Silkworm strains

Silkworm strains, C108, og and oq, were prepared as described in Chapter II. Another strain, og\textsuperscript{t}, was obtained from the Institute of Genetic Resources, Faculty of Agriculture, Kyushu University.

Crude enzyme extract preparation

The fat body of fifth instar larvae was dissected out and washed in cold 1x SSC. After blotted on filter paper, the tissue sample was frozen in liquid N\textsubscript{2} and kept at -80\degree C until protein extraction. The frozen sample was weighed, ground into powder in a mortar and added into 1 ml of extraction buffer per 0.1 g of the tissue. The extraction buffer consists of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 \mu M sodium molybdate, 10 \mu M FAD, 2 mM DTT, 5 \mu M leupeptin, 1 mM PMSF and 10 \mu g/ml trypsin inhibitor from soybean. After homogenization, the sample was centrifuged at 15,000g for 15 min at 4\degree C. The supernatant was recovered and concentrated 10 to 20 times by filtration with Centricon (UFC3LGC00, Millipore). The protein concentration of the crude extract was measured using a protein assay kit (BioRad) according to the manufacturer's protocol with bovine \gamma-globulin as a standard. The protein extract was kept at -20\degree C until use.
Native PAGE and activity staining

The protein sample was separated in 7.5% native PAGE at 4°C in a Laemmli buffer system without SDS (Laemmli, 1970). Thirty μg of the sample was loaded in each lane. Some gels were stained with Coomassie Brilliant Blue to confirm the equal concentration of the protein that was applied to the gel. Other gels were analyzed by the activity staining method of Seo et al. (1998) to detect XDH or AO: the gel was kept in 0.1 M potassium phosphate buffer (pH 7.5) for 5 min at room temperature, and then incubated at 30°C for 1 hour in staining buffer consisting of 0.1 M potassium phosphate buffer (pH 7.5), 1 mM substrate (hypoxanthine, xanthine or benzaldehyde), 0.1 mM phenazine methosulfate and 0.4 mM 3(4,5-dimethylthiazolyl-2)2,5-diphenyltetrazolium bromide. After the staining, gels were washed in distilled water and scanned into computer graphic files. In some experiments, 1 mM allopurinol was added to the buffers.

Amplification and sequencing of the \textit{BmXDH} cDNAs of the \textit{oq} mutant

Total RNA was extracted from the fat body of fifth instar \textit{oq}/\textit{oq} larvae. Primers for the reverse transcription were designed as follows to anneal to the 3' end of the \textit{BmXDH} cDNAs: 5'-
\[
\text{GTGAGTAGGTAGTGAATTTTTCAATCTTTT-3'}
\]
for \textit{BmXDHI} and 5'-
\[
\text{AAGAATTTGAGTCTAGGCAATTACATTCCA-3'}
\]
for \textit{BmXDH2}. Twenty μg of total RNA was mixed with 25 pmol of primer, and incubated at 70°C for 10 min and chilled on ice. The buffer containing the following substances were added to the RNA-primer mixture and the total volume was adjusted to 20 μl: 200 U of SuperScript™ II RNaseH-
Reverse Transcriptase (GIBCO BRL), 4 µl of 5x buffer supplied with the reverse transcriptase, 100 U of RNase inhibitor (TaKaRa), 0.1 M DTT and 40 mM dNTP mix. The reaction mixture was incubated at 48°C for 1 hour for the reverse transcription and then at 70°C for 15 min to inactivate the reverse transcriptase. RNase A and RNase H were added and the mixture was incubated at 37°C for 30 min to get cDNA solution. One µl of the cDNA solution was added as a template in PCR mixture to amplify the BmXDH cDNAs. The primers were as follows: 5'-TTCCCTCCCCAGTCTTTGTCTTTATTTGTGAC-3' and 5'-GTGAGTAGGTAATGTCAATCTTTTTCATCTOTTT-3' for BmXDH1, and 5'-ACGTAGTCTTGACAGTCTGATCTTTGATGTGTTC-3' and 5'-AAGAATTTGAGTCTAGGCAATTACATTCCA-3' for BmXDH2.

ExTaq (TaKaRa) was used in the amplification with the following cycle: 94°C for 3 min, (94°C for 30 sec, 55°C for 1 min and 72°C for 3.5 min) x 35 cycles and 72°C for 10 min. After expected PCR products were confirmed by agarose gel electrophoresis, they were purified with MicroSpin™ S-400 HR Columns (Amersham Pharmacia Biotech) and cloned into a plasmid vector, pGEM®-T Easy Vector (Promega). In order to eliminate the possibility of the misincorporation of nucleotides during RT-PCR, three reactions with each primer were carried out separately and one clone was picked up randomly from each reaction series. The three cDNA clones of each BmXDH gene were sequenced with ABI sequencers as described in Chapter I and compared to each other to obtain the original sequences.
Results

Activity staining of fat body XDH in native PAGE gels

XDH activity of the fat body in the mutant strains was analyzed by activity staining of native PAGE gels. The amount of the protein loaded to gels was confirmed by Coomassie Brilliant Blue stain of a gel, and it was shown that all samples contained equal amount of protein (Fig. 22). Only the $oq/oq$ and $og^f/+\,$ lanes showed weak 150-kDa bands while the others did not. Although it is not known where these bands originated from, they did not disturb the following experiments because their positions in the gel were different from those of the bands stained in the other experiments. Activity staining of XDH in the larval fat body samples is shown in Fig. 23; after the electrophoresis, the gel was stained with hypoxanthine as a substrate. The fat body of the control strain, C108, showed two bands, a major band at a higher position and a minor one at a lower position. Heterozygotes of the mutant strains, $oq/+\,$ (including $+/+\,$), $og/+\,$ and $og^f/+\,$ showed the major band of the same strength as that of C108. On the other hand, the major band was lost in all the mutant larvae, $oq/oq\,$, $og/oq\,$, and $og^f/oq\,$. It was clear that the major band corresponded to XDH enzyme because they were detected in the normal larvae but not in all the mutants that were shown to be XDH-deficient by other experiments (Hayashi, 1961; Tamura, 1977, 1983). The major band of the $og^f/+\,$ larva ran slightly faster than the other major bands, suggesting the existence of isozymes. The minor band was lost in $og/oq\,$ and $og^f/oq\,$, while it was detected in the $oq/oq\,$ mutant and heterozygotes of all strains although the signal strengths were varied from specimen to specimen. Since the $oq\,$ locus corresponded to the $BmXDH\,$ genes, the major band showed an enzyme that is encoded by one of the
Fig. 22. Native PAGE analysis of the protein of larval fat body. Thirty μg/lane of fat body protein was separated in a 7.5% native PAGE gel and stained with Coomassie Brilliant Blue.
Fig. 23. Activity staining of a 7.5% native PAGE gel with hypoxanthine. Thirty μg of protein of larval fat body was loaded in each lane. The black arrowhead indicates the position of the major band, and the white arrowhead indicates the minor band.
BmXDH genes. The minor band, which was active in the ogloq mutant, seemed to be the other BmXDH or other enzyme that has wide specificity of substrate. The oglog or ogf/ogt mutant did not show the minor band as well as the major one. The absence of the two bands indicated that the two mutants have abnormality in a factor that affects multiple enzymes.

The two bands shown in the activity staining with hypoxanthine exhibited certainly the activity of enzymes which dehydrogenate or oxidize xanthine because they were not detected when no substrate was added in the staining buffer (Fig. 24). To see the characteristics of the two bands as XDH, allopurinol was added in the buffer (Fig. 25). Allopurinol is a specific inhibitor of XDH, and it induces the phenocopy of the translucent-skin mutant when added to the diet of Bombyx larvae (Tamura, 1978). The major bands were much weaker with allopurinol than without it; the activity of the enzyme in the major band was inhibited by allopurinol. On the other hand, the minor bands were not affected by the inhibitor. When xanthine was added as a substrate instead of hypoxanthine, the signal of the major band was slightly weaker than that with hypoxanthine and the minor band was not detected (Fig. 26). These results showed that the major band was XDH and the minor band was another enzyme that oxidizes hypoxanthine as a non-specific substrate.

It was possible that the minor band was AO because it resembles XDH in the amino acid sequence. For the identification of the minor band, benzaldehyde was added as a substrate in the activity staining (Fig. 27). Contrary to the expectation, only the major band was stained weakly, while the minor band was not detected and did not seem to be AO. There are two possible reasons why the major band oxidized benzaldehyde: (1) AO protein was not able to be separated from XDH in the present electrophoresis or (2) the XDH in the major band oxidized benzaldehyde at a low rate.
Fig. 24. Activity staining of a 7.5% native PAGE gel with or without hypoxanthine. Thirty µg of protein of larval fat body was loaded in each lane. The black arrowhead indicates the position of the major band, and the white arrowhead indicates the minor band.
Fig. 25. Activity staining of a 7.5% native PAGE gel with hypoxanthine, and with or without allopurinol. Thirty μg of protein of larval fat body was loaded in each lane. The black arrowhead indicates the position of the major band and the white arrowhead indicates the minor band.
Fig. 26. Activity staining of a 7.5% native PAGE gel with xanthine. Thirty μg of protein of larval fat body was loaded in each lane. The black arrowhead indicates the position of the major band. The white arrowhead indicates the minor band detected in the control lane with hypoxanthine.
Fig. 27. Activity staining of a 7.5% native PAGE gel with benzaldehyde. Thirty μg of protein of larval fat body was loaded in each lane. The black arrowhead indicates the position of the major band. The white arrowhead indicates the minor band detected in the control lane with hypoxanthine.
The Northern hybridization showed that the transcription of the *BmXDH1* gene was decreased in the *oq* mutant, suggesting that the low transcription level caused the deficiency of XDH activity in the mutant (Chapter III). However, the activity staining on native PAGE gels showed that the *oq, og* and *ogt* mutants completely lacked the activity of XDH. Since the decrease of the *BmXDH1* mRNA was not able to explain well the complete absence of XDH activity, the cDNA of the *BmXDH* genes was recovered from the fat body of the *oq* mutant and the nucleotide sequences were determined.

The *BmXDH* cDNAs of the *oq* mutants were amplified from the total RNA of larval fat body by RT-PCR. To eliminate the possibility of misincorporation of nucleotides during reverse transcription or PCR, three different reactions were carried out to amplify the cDNA of each *BmXDH* gene. The PCR product of each reaction was cloned into plasmid vectors and one clone from each reaction series was sequenced (Figs. 28 and 29). In these figures, the nucleotide sequences of the three clones from different reactions were compared to each other to find the original sequence.

The cDNA sequence of the *BmXDH1* gene of the *oq/oq* mutant showed an 8-bp deletion at the position 3,242 (Fig. 28). This deletion caused replacements of 22 amino acid residues and a premature stop codon that would arrest the protein synthesis at the amino acid position 1,083 (Fig. 30). Since the deleted C-terminal portion included important domains that bind to a molybdenum cofactor, the truncated polypeptide of *oq* BmXDH1 was likely to lose the XDH activity. Although there were 13 replacements of amino acid in the N-terminal side of the deletion in BmXDH1 between C108 and *oq*, they are out of domains required for the enzyme activity.
Fig. 28. Comparison of the nucleotide and amino acid sequences of the \textit{BmXDHI} cDNA of C108 and the \textit{oq} homozygote. The nucleotides and amino acid residues which are identical to the genomic sequence are shown with dots, and gaps with dashes. The numbers at the right ends of the sequences are the positions of the nucleotides or amino acids from the beginning of the start codon. Synonymous substitutions are indicated with grey-shaded letters, and non-synonymous substitutions with black-shaded letters.
Fig. 28. (continued)
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Fig. 29. Comparison of the nucleotide and amino acid sequences of the BmXDH2 cDNA of C108 and the oq homozygote. The nucleotides and amino acid residues which are identical to the genomic sequence are shown with dots, and gaps with dashes. The numbers at the right ends of the sequences are the positions of the nucleotides or amino acids from the beginning of the start codon. Synonymous substitutions are indicated with grey-shaded letters, and non-synonymous substitutions with black-shaded letters.
Fig. 29. (continued)
| C108 nt | GCAAGCCCTATTTCTGACTTGAACCCAATTAATGGCTTGTAGTGCAGTACTTAATGTT | 1152 |
|         |                                                                 |     |
|         |                                                                 |     |
| C108 nt | TATAGCCTACGAAATGGGACGCGAACAAATAATCGATGAAAATTTCTTTAAAGGCTAC    |     |
|         |                                                                 |    1212 |
|         |                                                                 |     |
| C108 nt | A S P I S D L N P I L M A C S A V L N V                         |     |
|         |                                                                 |     |
|         |                                                                 |     |
| C108 nt | Y S T T N G S R Q I T I D E N F F K G Y                       |     |
|         |                                                                 |    404 |
|         |                                                                 |     |
| C108 nt | CGAAAGACAATATTAGAAGATGAGTTGATTTCCATATTACATTTCCACTT           | 1272 |
|         |                                                                 |     |
|         |                                                                 |     |
| C108 nt | T A T T A T T T A A T C T C A A C T C C A G G G T G A G G T A G   |     |
|         |                                                                 |     |
|         |                                                                 |     |
| C108 nt | V T A A F N V Q F E G N K V I K S K L C                       |     |
|         |                                                                 |     |
|         |                                                                 |     |
| C108 nt | G G M G P T T L L A S K S S K M L L G                         |     |
|         |                                                                 |     |
|         |                                                                 |     |
| C108 nt | A A A A T T G G A T C A T G A A A A C C T C A G G C G G T T T T C |     |
|         |                                                                 |     |
|         |                                                                 |     |
| C108 nt | L E F S V P G G M A E Y R K S L C L S L                       |     |
|         |                                                                 |     |
|         |                                                                 |     |
| C108 nt | T T C T T T A A T T T C T T C T T C A A C T G T C A A A A G T A A A T T A G A T A T A T C A A A C G G A A A G G C T C A A |     |
|         |                                                                 |     |
|         |                                                                 |     |
| C108 nt | Y F E I R N S G E V D A L G K P L P H A                       |     |
|         |                                                                 |     |

**Fig. 29. (continued)**
Fig. 29. (continued)
Fig. 29. (continued)
Fig. 29. (continued)
TTCAATGTTTCGCTGCTGAAAGGTGCTCCAAACCCACGTGCAGTATATTCTTCTAAGGCC

\[ \text{F N V S L L K G A P N P R A V Y S S K A} \]

ATCGGGGAAGCACAATGCTTTAGCCTCCAGTTTTCTTCTTGCCACATCAAGAAGAAATA

\[ \text{I G E P P L F L A A S V F F A I K E A I} \]

ATGGCCGCTCGTCGAAAACGACAGTGGGAGCCGTGAACTGGATGGATCACCTGCGACT

\[ \text{M A A R S D S G V P V E F E L D A P A T} \]

TGTGAACGTATTCGAATGTCTATGTGAAGACGACATCACTCTGAGTTAAGCCAACGTGA

\[ \text{C E R I R M S C E D D I T L Q V K P T V} \]

AAACGTATTGAGTTCGAGAATGTAATTGCCTAG

\[ \text{K R I G V P W N V I A *} \]

**Fig. 29. (continued)**
Fig. 30. Diagram of amino acid replacements between BmXDHs of C108 and the \textit{oq} homozygote shown in Figs. 28 and 29. Amino acid replacements are indicated with white arrowheads. BmXDH1 of the \textit{oq} mutant is truncated because of the premature stop codon. Domains required for the enzyme activity are also shown.
On the other hand, the BmXDH2 cDNA of oq was almost the same as that of C108 and there are only three amino acid replacements (Figs. 29 and 30). There were 32 synonymous substitutions of nucleotide in BmXDH1 of oq, while 12 in BmXDH2.
DISCUSSION

XDH activity of the silkworm has been analyzed by enzyme assay with the crude extract of protein added to reaction buffers (Hayashi, 1960, 1961; Tamura, 1977, 1983). This method had two lines of ambiguity: (1) it could not distinguish low activity from background signal, and (2) there was little consideration that multiple enzymes contribute to the activity in a mass. The new method described in this chapter, i.e., the activity staining of native PAGE gels, solved these problems. It showed signals as bands on the gel, making it easy to distinguish each signal from others and the background. The present experiments showed that there were at least two enzymes that were able to oxidize hypoxanthine and the major band was completely lost in XDH-deficient mutants. The major band represented the main source of XDH activity because it oxidized both hypoxanthine and xanthine, and the activity was inhibited by allopurinol, a specific inhibitor of XDH.

On the other hand, the minor band found in the activity staining with hypoxanthine seemed to be different from typical XDHs; it was not inhibited by allopurinol. There are two criteria explaining why the minor band was resistant to allopurinol. The first is that the minor band represented a different enzyme, e.g., AO, which has broad substrate specificity and oxidizes hypoxanthine; AO is resistant to allopurinol. The other is that the minor band represented BmXDH2. In some mutant XDH genes of A. nidulans, single amino acid replacements make XDH be resistant to allopurinol and less active than wild-type XDH in the absence of the inhibitor (Glatigny et al., 1998). One of the Aspergillus mutant XDH has an amino acid sequence "SSTATS" at a MoCo binding motif instead of "SSTAAS" found in normal XDH. This motif is involved in substrate specificity and is not conserved in AOs (Glatigny et al., 1998).
BmXDH2 has "TATAAS" in this motif while BmXDH1 has "SATAAS". All XDHs that have been sequenced have a serine residue at the first position of the motif but BmXDH2 has a threonine residue. The difference of the first residue might change the specificity of substrates and inhibitor. It is interesting to identify the origin of the two bands in the activity staining by purification and sequencing of the proteins, or by expressing the BmXDH genes and analyzing the product of each gene on native PAGE gels. It is also important to test the possibility of XDH heterodimer in the silkworm. XDH is known to act as a homodimer in other tested organisms, which have only one XDH gene. Since the silkworm is the only one organism that is proved to have tandemly duplicated XDH genes, the analysis of enzymatic action of XDH heterodimer, if exists, will give suggestive information. Recently, a heterodimer of AO, as well as two homodimers, was found in A. thaliana, which has at least two active AO genes (Akaba et al., 1999).

Two mutant loci, og and oq, have been known to be related to XDH deficiency but the difference has not been clear (Tamura, 1977, 1983). In this chapter, the minor band in stained gels showed the biochemical difference between these loci. The mutants of og and og' strains did not have the minor band while the oq mutant conserved it. Because the oq locus corresponds to a structural gene of XDH as shown in previous chapters, it is natural that the oq mutant lost only one band in the PAGE gel, i.e., the oq gene encoded the protein in the major band. The og locus was likely to correspond to an enzyme related to the biosynthesis of MoCo. In D. melanogaster, three XDH-deficient mutants, cin, lxd and mal, are known to be deficient in MoCo biosynthesis (Kamdar et al., 1997). They lack the activity of AO as well as that of XDH. The cin gene has been cloned and shown to be homologous to MoCo biosynthesis enzymes of other organisms, rat, A. thaliana and Escherichia coli.
The og gene of the silkworm may be homologous to one of the Drosophila genes, cin, lxd or mal.

The nucleotide sequences of the BmXDH cDNAs in the fat body of the og/og larva gave a definite explanation of the cause of the og mutant phenotype. BmXDH1 in the og mutant encoded a truncated XDH protein that had incomplete binding sites of MoCo while BmXDH2 was almost intact. These results, accompanied by the Northern blotting, show that the XDH-deficiency of the og mutant larvae is due to the abnormal translation of BmXDH1. This explains the complete loss of a XDH band in og/og larvae in native PAGE gels. Both synonymous and non-synonymous substitutions of nucleotides were larger in number in BmXDH1 than in BmXDH2 probably because there was no selective pressure on the non-functional BmXDH1 gene of the og mutant. If the difference of the substitution rate reflects the function of the BmXDH genes, BmXDH2 gene seems functional in spite of the low amount of the transcripts.
Summary

XDH activity in the mutants og, og\(^l\) and oq was analyzed by the activity staining of native PAGE gels. In these experiments, fat body protein showed two XDH bands, which were not able to be separated in the previous methods. One band, the major band, was very clear and the other band, the minor band, was faint. The oq mutants lacked the major band, while the og and og\(^l\) mutants lacked both. Allopurinol, a specific inhibitor of XDH, decreased the signals of the major band but not those of the minor band. Xanthine and benzaldehyde stained the major band but not the minor band. These results suggested that the major band represented XDH and the minor one was an enzyme other than XDH or AO. As both bands were lost in the mutants of og and og\(^l\), it was possible that the minor band was an enzyme which requires molybdenum cofactors (MoCo) and the og locus corresponded to a gene in the MoCo biosynthesis. The cDNA sequence of BmXDHI of the oq mutant had an 8-bp deletion which would cause premature termination of transcription. Several sites for MoCo binding were lost in the oq mutant BmXDHI. This result explained the complete lack of the major band in the oq mutant. The BmXDH2 cDNA seemed to be intact in the oq mutant.
GENERAL DISCUSSION

More than 20 mutants of translucent-skin silkworm larvae have been isolated and analyzed. The larval hypodermis of the mutants fail to accumulate uric acid that makes the skin opaque and white in normal larvae. There are two types of translucent-skin mutants: (1) mutants which are deficient in accumulation of uric acid in the hypodermis and (2) mutants which are incapable of synthesizing uric acid (Hayashi, 1961; Tamura, 1977, 1983; Tamura and Sakate, 1983). The first group include \( o_a, o_{al}, o_{bi}, o_c, o_d, o_k, o_r, o_s, o_{v}, o_{w} \) and \( w-Joe \). The other group consists of \( o_g, o_{gl} \) and \( o_q \), which were analyzed in this report. As shown by genomic Southern hybridization, Northern blotting, activity staining on native PAGE gels and cDNA sequencing, the \( o_q \) locus corresponded to the \( BmXDH1 \) gene (Fig. 31). The deficiency in XDH activity was caused by 8-bp deletion in the \( BmXDH1 \) gene followed by premature termination of the translation. On the other hand, the role of the \( BmXDH2 \) gene is still unclear. Low amount of the \( BmXDH2 \) transcript seemed to fail to compensate the deficiency of \( BmXDH1 \) in the \( o_q \) mutants although the deduced amino acid sequence of the \( BmXDH2 \) gene showed no apparent abnormality especially in the conserved regions. It is possible that the transcription level of the \( BmXDH2 \) was too low to give detectable XDH activity, or that the \( BmXDH2 \) protein has little or no activity as XDH.

The analysis of each \( BmXDH \) protein artificially expressed in bacteria or insect cells will help the understanding of the difference of the \( BmXDH \) proteins. The protein expression experiments will also give an important evidence of the inactivity of the truncated \( BmXDH1 \) protein of the \( o_q \) mutant.

The role of the \( o_g \) gene in the formation of active XDH remains to be uncertain. However, the activity staining of XDH from the \( o_g \) or \( o_{gl} \)
Fig. 31. Diagram of the relationship between translucent-skin mutations and the process of uric acid accumulation. The mutations are shown with grey boxes. The arrow with a black arrowhead indicates the synthesis or the transportation of substances, and the arrow with a white arrowhead indicates the effect of the substances.
mutants suggested a possible role of the \( og \) gene: molybdenum cofactor biosynthesis. In \( D. \ melanogaster \), three genes, \( cin, mal \) and \( lxd \), are known to act in the biosynthesis of molybdenum cofactor, which is required by XDH, AO and pyridoxal oxidase (Kamdar et al., 1997). The mutants of these genes have no activities of the three enzymes. In the activity staining experiments, normal larvae had two XDH bands, whereas the \( og \) or \( og^t \) mutants did not have either band and the \( og \) mutant, which corresponded to the structural gene of XDH, lost only one. These results show the similarity of the \( og \) gene and the \( Drosophila \) MoCo biosynthesis genes, suggesting the relationship between the \( og \) gene and MoCo synthesis. The cloning of the \( og \) gene will be necessary to identify it.

The phylogenetic tree of animal XDHs indicates that the duplication of XDH genes occurred early in the lepidopteran evolution. Duplication of other genes has been reported in lepidopteran species, e.g., aminopeptidase genes (Chang et al., 1999). The analysis of XDH gene duplication in other insects, including lepidopterans, will help the understanding of gene evolution in insects. The analysis of intron sites of lepidopteran XDH genes will also give important information on the gene evolution. Tarrío et al. (1998) studied intron sites in dipteran XDH genes and showed the possibility of new intron insertion during the evolution. Since the XDH introns are conserved between vertebrates and insects, the XDH gene will be a good model to study intron evolution.

The XDH-deficient mutants of the silkworm, \( og \) and \( og \), may serve as a tool for the study of the biological role of uric acid. It has long been known that uric acid is the main substance to excrete nitrogen waste in insects, birds and terrestrial reptiles. If insects excrete urea instead of uric acid, they will require much water to avoid high osmotic pressure because urea is highly soluble in water. On the other hand, uric acid is almost insoluble in water and little water is required to excrete it. The bad effect
of low activity of XDH in the silkworm mutants og and oq has been explained by the toxic effect of accumulated hypoxanthine or xanthine. Many mutants die before eclosion; og is female sterile, while ogf and oq are both female and male sterile (Sasaki, 1937). As described in Introduction, it was reported that uric acid plays an important role as an antioxidant to remove active oxygen (Ames et al., 1981). The ry mutant of D. melanogaster, which is deficient in XDH, has a shorter life span and is more sensitive to active oxygen, caused by insecticide or high temperature, than the normal fruit fly (Shepherd et al., 1989; Hilliker et al., 1992). However, it is still difficult to prove the role of uric acid as an antioxidant in vivo because the experiments with the ry mutant were not able to exclude the possibility of the toxicity of hypoxanthine or xanthine. Since Bombyx larvae are larger than fruit flies, it will be easy to analyze the biological function of uric acid in vivo by injecting it in larvae or pupae of XDH-deficient silkworm mutants and examining their viability or fertility.

The BmXDH1 gene and the oq mutant can be used as a selection marker and a recipient, respectively, to produce transgenic silkworms. The system to get transgenic silkworms has long been expected because it will help effectively the analysis of mutants, the study of gene functions and the production of foreign proteins. Several vectors have been studied and some were shown to be capable of constructing transgenic silkworms; a transposon vector originated from piggyBac for random insertion, a baculovirus vector for homologous recombination, and a pseudotyped retroviral vector for the transformation of somatic cells (Yamao et al., 1999; Tamura et al., submitted; Kōmoto et al., submitted). In most studies of the vectors, green fluorescent protein (GFP) gene is used as a selection marker because GFP can be detected easily in living animals without any fixation or chemical stainings. However, other markers
derived from endogenous silkworm genes will contribute to the development of gene introduction technique because their expression will be able to be seen without any special apparatus, e.g., the fluorescent microscope which is necessary for the GFP selection. In addition, the long wavelength UV light, which is often used to excite the GFP protein, can be harmful to the silkworm, while endogenous marker genes will not have bad influence. As shown in this report, the $oq$ mutant phenotype is caused by the deficiency in the $BmXDH1$ gene. If a vector containing $BmXDH1$ as a marker is introduced into the $oq$ mutant, transgenic $Bombyx$ larvae will have opaque skin and will be distinguishable from non-transgenic larvae which have translucent skin. Although the $oq/oq$ moths are sterile and it has been difficult to mate mutant homozygotes to get mutant silkworms, the injection of bovine XO, which can be obtained from commercial source, is able to recover the fertility and makes it easy to obtain $oq/oq$ eggs (Tamura et al., 1999). Combining the $oq$ mutants, the $BmXDH1$ gene and the XO injection, a new system to create transgenic silkworms will be developed.
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