REDUCTASES REDUCING PLANT HEMOGLOBINS AND POSSIBLE MECHANISM OF THEIR ACTION

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Metleghemoglobin reductases from nodules of yellow and blue lupines and garden pea were obtained in homogeneous state, some of their properties were studied. Presence of two isoforms of enzyme in nodules of both lupine species was shown. The comparison of properties of studied metleghemoglobin reductases from different legume plants was made. Possible mechanisms of their functioning in nodules are discussing.

For normal run of nitrogen fixation process optimal oxygen conditions in nodules of legume plants are necessary. One of the most important parts of system regulating these conditions is special plant hemoprotein—leghemoglobin. It is possible to bind oxygen and to form oxygenated LbO_2 form only in physiologically active reduced state.

Two ways of supporting leghemoglobin in reduced state are known: enzymatic (reducing by metleghemoglobin reductase) and non-enzymatic (reducing by indolyl-acetic acid [1], reduced flavins [2], cysteine, ascorbic acid and low molecular weight component B [3]). Thanks to presence of several enzymatic and non-enzymatic reductants in nodule cytosol the considerable removal of $Lb^{3+} \rightleftarrows Lb^{2+}$ balance to formation of reduced hemoprotein is observing, norm of physiological reaction of symbiotic nitrogen-fixing system is extending, and optimal regime for nitrogen fixation are supporting at widely changing conditions. Our calculations show that enzymatic reduction makes the main contribution to Lb supporting in reduced state.

Process of Lb enzymatic reduction was firstly described in A.N.Bach Institute of Biochemistry in the laboratory of Prof. W. L. Kretovich [4]. Till now MlbRs from nodule cytosol of yellow lupine (*Lupinus luteus* L.) [5], soya (*Glycine max* (L.) Merr.) [6] and cowpea (*Vigna unguiculata* L.) [7]. It was shown that lupine MLbR was a monomer with molecular weight ~ 60 kDa, and soybean and cowpea ones are dimers with molecular weight of native enzyme ~ 110 kDa (weight of subunit 55 kDa). In lupine nodules non-heme iron-protein possible to reduce Lb^{3+} was also found [8, 9]. All MLbRs studied were flavoproteins and could to transfer electrons from reduced pyridinenucleotides to oxidized hemoproteins.

Primary structure of soybean and cowpea enzymes was determined [7, 10]. It was shown that these MLbRs contained NADH- and FAD-binding domains and disulfide active centre. Tertiary structure is not determined not for one of MLbRs.

The purpose of our work was to study of process of Lb enzymatic reduction and discussing of probable mechanisms of this process *in vivo*.

Methods of Study

Root nodules of yellow lupine (*Lupinus luteus* L.), cv. Bryansky-27 and blue lupine (*Lupinus angustifolium* L.) cv. Bryansky-109 were used. Lupine plants were inoculated with *Rhizobium lupini* inoculating bacteria (effective strain 359a from the collection of All-Russian Research Institute of Biotechnology). Root nodules of garden pea (*Pisum sativum* L.) cv. Finae were also used. Pea plants were inoculated with *Rhizobium leguminosarum* inoculating bacteria (effective strain bv. *Viceae* CIAM 1026 from the collection of All-Russian Research Institute of Agricultural Microbiology). Plants were grown in field conditions of Moscow region.

Nodules were harvested at the budding - beginning of flowering stage of plant growth because it was shown [11] that MLbR activity and Lb content were maximal at this stage.

Isolation and determining of MLbR activity and Lb content were made according to methods described in [12].

Optical spectra were recorded at the spectrophotometer Hitachi-557 (Japan). Fluorescence spectra were recorded at the spectrofluorimeter Hitachi MPF-4 (Japan).

Reagents used: DEAE-Toyopearl 650M—Toyo Soda (Japan); ampholines—LKB-Pharmacia (Sweden); 2,6-dichlorophenolindophenol, thiasolyl blue, phenylmethylsulfonylfluoride, Coumassie blue G-250 and R-250, kits of marker proteins for gel-filtration and electrophoresis, Ultrogel AcA 44, Tris, glycerol—Serva (Germany); NADH, acrylamide, N,N-methylenebisacrylamide—Reanal (Hungary) (reagent for electrophoresis were recrystallised); glycine—ICN (USA), *o*-phenantroline, polyvinilpirrolydone, sucrose, methanole, ethanole, K_2HPO_4, KH_2PO_4, NaCl, KCl, $MgCl_2$, $(NH_4)_2SO_4$—Reachim (Russia) of purest grade.

Results and Discussion

Metleghemoglobin reductases from yellow (*Lupinus luteus* L.) and blue (*Lupinus angustifolium* L.) lupines and

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Abbreviations: Lb, Lb^{2+}, Lb^{3+}, and LbO_2 are leghemoglobin, reduced, oxidized and oxygenated leghemoglobin, respectively; MLbR is metleghemoglobin reductase; IEP is isoelectric point.

from garden pea (*Pisum sativum* L.) were obtained in homogeneous state. Comparison of their properties with those of soybean and cowpea enzymes was made.

The comparison of some properties of MLbRs from different legume plants is shown in Table 1. Two isoforms of enzyme with different properties were found in nodules of both lupine species. One of enzymes—monomeric FAD-containing protein with molecular weight 62 kDa inhibiting by reagents for SH-groups. The second was homodimer with molecular weight of subunit 66 kDa similar to soybean and cowpea reductases. The monomeric MLbR was more active.

Table 1

Comparison of properties of MLbRs from different legume plants

Properties	Yellow lupine	Blue lupine	Garden pea	Soya [6]	Cowpea [7]
Presence of isoforms	yes 2	yes 2	no	no	no
MW (kDa) native subunit	120 62 66 62	120 60 66 60	120 59	110 54	110 55
Presence of FAD	yes	yes	yes	yes	yes
IEP	6.14 5.83	6.18 5.88	5.65	5.6*	—

* from article [6] as average IEP of multiple forms of enzyme.

Further studies of MLbR from yellow lupine showed the presence of non-heme iron in dimeric enzyme (2 atoms per enzyme molecule) [13]. At the same time 62 kDa protein did not contain metals. Enzymatic activity of MLbR containing non-heme iron was inhibited by *o*-phenantroline. It indicated the presence of this metal in active centre of enzyme. Probably dimeric MLbR is the non-heme iron-protein formerly found in legume nodules.

We also studied the secondary structure of MLbR with the method of CD-spectroscopy [13]. It was large difference in secondary structures of proteins with molecular weights of 66 and 62 kDa. In spite of similarity of irregular regions, there are reliable differences in α-helix and β-structure organization of proteins.

Enzyme from pea nodules was similar to soybean and cowpea reductases (Table 1). We have to note that from nodules of these three legume species only dimeric MLbR was isolated.

Thus, according to our and literary data it is possible to make conclusion that two isoforms of MLbR catalyzing reaction of Lb reduction *in vivo* can exist in nodule cytosol of legume plants.

It is known that oxidation-reduction reactions catalyzing by flavin- and SH-containing enzymes usually are bimolecular. Stechiometry of Lb reduction reaction is 2 to 1:

$$\mathrm{NADH} + \mathrm{H}^+ + 2\mathrm{Lb}^{3+} \rightarrow 2\mathrm{Lb}^{2+} + \mathrm{NAD}^+ + 2\mathrm{H}^+.$$

Evidently MLbR works as two-electron acceptor when reacts with reduced pyridinenucleotides and as one-electron donor during Lb^{3+} reduction. This supposition agrees with data of presence of pare of SH-groups in active centre of enzyme (in case of SH- and flavin-containing soybean and cowpea MLbRs) [7, 10] or of pare of Fe-S clasters (in case of Fe-S flavin-containing lupine MLbR) [9, 13]. We can suppose that after binding of $\mathrm{NADH} + \mathrm{H}^+$ two electrons are passing through intermolecular way to oxidized FAD^+, reducing it to $\mathrm{FAD}{*}\mathrm{H}_2$. Indirect proof of our supposition is the fact that standard red-ox potential of protein-bound FAD (~ -0.09 V) has intermediate position in the line of potentials: pyridinenucleotides (-0.28 V) $\rightarrow$ Lb($+0.24$ V). Spectrophotometric studies of MLbR in conditions expelling oxidation [5] also support this supposition. Absorbance spectrum of native reductase has maximum in the 450 nm region corresponding to FAD^+. $\mathrm{FAD}{*}\mathrm{H}_2$ can later secure reduction either SH- or Fe-S active centre, from which electron is transferring to Lb^{3+}.

Enzymatic reduction of mammal Hb is going with participation of electron carrier from methemoglobin reductase to Hb. It was proved that cytochrome b_5 is this carrier [14]. Native electron carrier during Lb^{3+} reduction in nodules is not yet found. In *in vitro* experiments Lb^{3+} reduction could be made with use of methylene blue [11] or cytochrome b_5 [15] as intermediate electron carriers. The rate of Lb^{3+} reduction by purified MLbR *in vitro* was much lower at the absence of electron carriers [16]. Because of it we proposed the idea of possible participation of intermediate electron carriers in process of enzymatic Lb^{3+} reduction *in vivo* [15]. The same supposition was made in article [3]. At the same time the direct transfer from enzyme to oxidized hemoprotein is not excluded.

Thus, the scheme of possible mechanism of enzymatic Lb^{3+} reduction *in vivo* can be shown in such way:

1. $\mathrm{NADH} + \mathrm{H}^+ + {}^+\mathrm{FAD}\text{–}\mathrm{MLbR}\text{–}\mathrm{A}^{2+} \rightarrow$
   $\rightarrow {}_2\mathrm{H} * \mathrm{FAD}\text{–}\mathrm{MLbR}\text{–}\mathrm{A}^{2+} + \mathrm{NAD}^+$,
2. ${}_2\mathrm{H} * \mathrm{FAD}\text{–}\mathrm{MLbR}\text{–}\mathrm{A}^{2+} \rightarrow {}^+\mathrm{FAD}\text{–}\mathrm{MLbR}\text{–}\mathrm{A}^0 + 2\mathrm{H}^+$,
3. ${}^+\mathrm{FAD}\text{–}\mathrm{MLbR}\text{–}\mathrm{A}^0 \xrightarrow[-{}^+\mathrm{FAD}-\mathrm{MLbR}-\mathrm{A}^{2+}]{\text{with carrier or without it}} \begin{matrix}\mathrm{Lb}^{3+}\\ \downarrow\uparrow \\ \mathrm{Lb}^{2+}\end{matrix}$,

where A^0 and A^{2+} are reduced and oxidized active centres of enzyme.

This scheme is rather conditional. For exact qualitative and quantitative description of mechanism of enzymatic Lb^{3+} reduction further studies of MLbR structure and functioning are necessary.

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References

1. Puppo A. and Rigaud J. (1975) *Physiol. Plantarum*, **35**, 181–185.
2. Becana M., Salin M.L., Ji L., and Klucas R.V. (1991) *Planta*, **183**, 575–583.
3. Becana M. and Klucas R.V. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 7295–7299.
4. Melik-Sarkissyan S.S., Bashirova N.F., Zauralova N.O., and Kretovich W.L. (1976) *Biokhimia (Biochemistry)*, **41**, 1330–1333.
5. Golubeva L.I., Topunov A.F., Goncharova S.S., Aseeva K.B., and Kretovich W.L. (1988) *Biokhimia (Biochemistry)*, **53**, 1712–1717.
6. Ji L., Wood S., Becana M., and Klucas R.V. (1991) *Plant Physiol.*, **96**, 32–37.

7. Luan P., Arechaga-Ocampo E., Sarath G., Arredondo-Peter R., and Klucas R.V. (2000) *Plant Science*, **154**, 161–170.
8. Borodenko L.I., Genina O.S., Zhiznevskaya G.Ya., and Izmailov S.F. (1990) *Fiziologiya Rastenij (Plant Physiology)*, **37**, 1131–1137.
9. Zhiznevskaya G.Ya., Borodenko L.I., Kudryavtseva N.N., Lowe D.J., Vanin A.F., and Izmailov S.F. (1999) *Fiziologiya Rastenij (Plant Physiology)*, **46**, 738–741.
10. Ji L., Becana M., Sarath G., and Klucas R.V. (1994) *Plant Physiol.*, **104**, 453–459.
11. Bashirova N.F., Melik-Sarkissyan S.S., and Kretovich W.L. (1979) *Biokhimia (Biochemistry)*, **44**, 1240–1245.
12. Shleev S.V., Rozov F.N., and Topunov A.F. (2000) *Prikl. Biokhim. Mikrobiol. (Appl. Biochem. Microbiol.)*, (in press).
13. Shleev S.V., Shubin V.V., Voevudskaya N.V., Vanin A.F., and Topunov A.F. *Biokhimia (Biochemistry)*, (in press).
14. Passon P.G. and Hultquist D.E. (1972) *Biochim. Biophys. Acta*, **275**, 62–73.
15. Topunov A.F., Melik-Sarkissyan S.S., Lysenko L.A., Kretovich W.L. (1982) *Biokhimia (Biochemistry)*, **47**, 442–445.
16. Golubeva L.I., Topunov A.F., Talysin V.V., and Kretovich W.L. (1987) *Dokl. Akad. Nauk SSSR (Proc. USSR Academy of Sciences)*, **294**, 1489–1492.