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The origin of pyruvate:NADP$^+$ oxidoreductase in mitochondria of *Euglena gracilis*[1]

Masami Nakazawa[a], Hiroshi Inui[a,*], Ryoichi Yamaji[a], Takuya Yamamoto[a], Shigeo Takenaka[b], Mitsuhiro Ueda[a], Yoshihisa Nakano[a], Kazutaka Miyatake[a]

Euglena gracilis, a protist having chloroplasts, has unique mitochondria, in which the common pyruvate and 2-oxoglutrate dehydrogenase multienzyme complexes are absent [1]. In the mitochondria, 2-oxoglutrate is converted to succinate through succinate semialdehyde by the sequential reactions of 2-oxoglutrate decarboxylase and succinate semialdehyde dehydrogenase. In contrast, pyruvate is oxidized in a CoA-dependent reaction to form acetyl-CoA and CO_2 by the action of a single enzyme, pyruvate:NADP$^+$ oxidoreductase (PNO) (EC 1.2.1.51), with NADP$^+$ (but not NAD$^+$) as an electron acceptor. It is suggested that these three enzymes participate in the respiratory metabolism of this organism. PNO, in contrast to the other two enzymes, has not yet been found in any other organisms. These unique characteristics of *Euglena* provide insight regarding the origin of mitochondria. In this communication, we propose that PNO was evolved from pyruvate:ferredoxin oxidoreductase (PFO) by linking with a flavoprotein with both FMN and FAD at the C-terminal side by gene fusion.

To address the origin of PNO, we cloned cDNA encoding this enzyme and analyzed it. The PNO cDNA (5861 bp) had an open reading frame encoding a polypeptide with 1803 amino acid residues. The N-terminal amino acid in the mature form of this enzyme (Thr [1]) was found at the position of 38 in the deduced amino acid sequence of the cDNA. It is thus indicated that the mature form of PNO, a homodimeric protein, consists of 1766 amino acid residues per subunit (calculated subunit molecular weight: 195 536), and the precursor form of this enzyme has a signal sequence with 37 amino acid residues for targeting to mitochondria.

We have reported that limited proteolysis of PNO with trypsin gives two functionally active fragments [1]. One of the two fragments, derived from the N-terminal side of this enzyme, retains a homodimeric structure, and is still capable of catalyzing the CoA-dependent oxidative decarboxylation of pyruvate when an artificial electron acceptor, such as methyl viologen, is substituted for NADP$^+$. Another fragment (the C-terminal fragment) is a monomeric peptide and retains an electron-transfer activity from NADPH to methyl viologen or other artificial electron acceptors. In the deduced amino acid sequence of the PNO cDNA, the N-terminal amino acid of the C-terminal fragment (Ala [1]) was found at the position of 1240. The amino acid sequence of the N-terminal fragment of PNO (amino acid position in the deduced amino acid sequence of the cDNA: 38–1239) closely resembled the sequences of homodimeric PFOs found in amitochondriate eukaryote *Entamoeba histolytica* (53% positional identity) and *Trichomonas vaginalis* (44%), and in eubacterium *Rhodospirillum rubrum* (44%), *Klebsiella pneumoniae* (49%) and *Desulfovibrio africanus* (53%). Similar to these homodimeric PFORs [2], this result shows that the N-terminal part of PNOR likely contains in each subunit one TBP, two ferredoxin-type [4Fe–4S] clusters, and one [4Fe–4S] cluster bound to an atypical cysteine-containing sequence motif. In contrast, the C-terminal fragment of PNO (amino acid position: 1240–1803) showed structural similarity to mammalian NADPH-cytochrome P450 reductase (positional identity to rat enzyme: 28%) and to bacterium NADPH-sulfite reductase α subunit (positional identity to *Salmonella typhimurium* enzyme: 21%). This finding suggests that the C-terminal part of PNOR, as well as NADPH-cytochrome P450 reductase and NADPH-sulfite reductase α subunit [3], has FMN and FAD (each one per subunit).

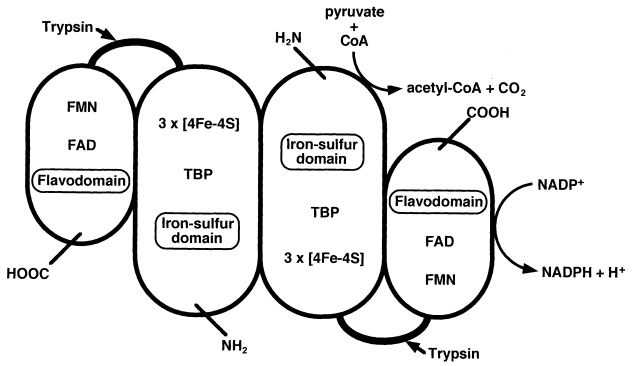

Fig. 1. A structural model and proposed reaction mechanism of PNO. ←Trypsin: trypsin-sensitive hinge region; NH_2: N-terminal; COOH: C-terminal.

It is thus suggested that each subunit (about 195 kDa) of PNO, a homodimeric protein, consists of two functional components, Fe–S domain (about 130 kDa) and flavodomain (about 65 kDa); the Fe–S domain locates at the N-terminal side and is linked with the flavodomain by a trypsin-sensitive hinge region (Fig. 1). The reaction mechanism of this enzyme is thought as follows: On the Fe–S domain, pyruvate is oxidized in a CoA-dependent reaction to form acetyl-CoA and CO_2 by the cooperation of TBP and [4Fe–4S] clusters. Two electrons formed by the pyruvate oxidation in the Fe–S domain are once transferred to the flavodomain with FMN and FAD as redox centers, then accepted by NADP$^+$.

In archaea, most anaerobic eubacteria and amitochondriate eukaryotes, PFO acts for the oxidative decarboxylation of pyruvate to form acetyl-CoA, and the two electrons formed are accepted by ferredoxin with a more negative redox potential than that of NAD$^+$ [2]. This is in contrast to most respiratory organisms having the pyruvate dehydrogenase multienzyme complex. On the basis of subunit structure, PFOs are divided into homodimeric, heterodimeric and heterotetrameric types. These three types of PFO are phylogenetically close and their evolution is explained by the rearrangement and eventual fusion of four ancestral genes. Amitochondriate eukaryotes have homodimeric PFO, and this enzyme plays a central role in anaerobic energy metabolism [4,5]. In particular, in amitochondriate eukaryotes having hydrogenosomes (the double-membrane-bound, H_2- and ATP-producing organ-

0014-5793/00/$20.00
PII: S0014-5793(00)01891-3

elles), such as *Trichomonas*, PFO is located in the organelles and participates, together with hydrogenase, in the formation of acetate and H_2 from pyruvate, yielding ATP by substrate-level phosphorylation.

On the basis of this evidence, we propose that PNO was evolved by linking with a homodimeric PFO and a flavoenzyme (which is the same origin as NADPH-cytochrome P450 reductase and NADPH-sulfite reductase α subunit) by gene fusion. In other words, the ferredoxin-type enzyme was converted to the $NADP^+$-dependent one by linking covalently with a flavoprotein with both FMN and FAD at the C-terminal side. In addition, it is thought that this conversion was necessary to make it possible to participate in the respiratory metabolism in the mitochondria of *E. gracilis*. In the hydrogen hypothesis for the first eukaryote, mitochondria and hydrogenosomes are proposed to originate from a common ancestor [5]. Thus, the occurrence of PNO in *Euglena* mitochondria is interesting in understanding the relationship between mitochondria and hydrogenosomes.

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*Corresponding author. Fax: (81)-722-50 7318.
E-mail: inui@biochem.osakafu-u.ac.jp

[1] The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/ GenBank nucleotide sequence databases with the accession number AB021127.

[a] *Department of Applied Biological Chemistry, Osaka Prefecture University, Sakai, Osaka 599-8531, Japan*
[b] *Laboratory of Nutrition and Food Science, Hagoromo-Gakuen College, Sakai, Osaka 592-8344, Japan*

FEBS 24007

Disulfide bridges of Ergtoxin, a member of a new sub-family of peptide blockers of the *ether-a-go-go*-related K^+ channel

C. Bottiglieri[a], L. Ferrara[a], M. Corona[b], G.B. Gurrola[b], C. Batista[b], E. Wanke[c], L.D. Possani[b,*]

A new class of peptides named Ergtoxin (ErgTx), which block ERG-K^+-channels of nerve, heart and endocrine cells, was isolated from the venom of the Mexican scorpion *Centruroides noxius* [1]. Because ErgTx showed a significantly different amino acid sequence from those of previously reported

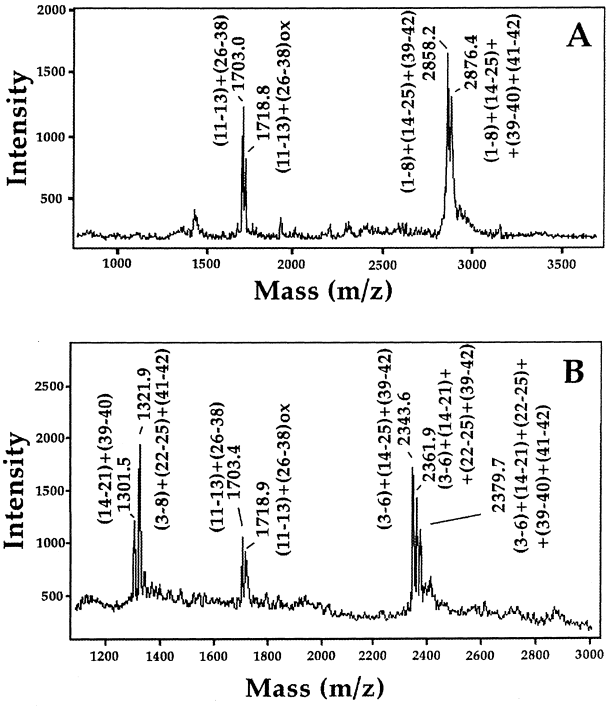

Fig. 1. MALDI-MS analysis of ErgTx digests. Native protein was digested with trypsin (A) followed by endopeptidase AspN (B). Signals corresponding to the disulfide-bridged peptides are indicated; each signal is assigned to the corresponding peptide pair.

K^+-channel specific scorpion toxins [2], it was important to determine the disulfide bridges and compare it with the others. Due to the presence of two cysteines in immediate vicinity (positions 23 and 24) it was not possible to determine its disulfide pairing using classical biochemical methods, and a thorough analysis by mass spectrometry was performed [3,4]. During this procedure, we discovered that two amino acid residues of the peptide used for our experiments [1] were not the same. There is an extra disulfide bridge and consequently two of the previous reported residues (a glutamine and a proline residue) are in fact cysteines. These results were obtained by direct sequencing, mass spectrometry analysis of native versus alkylated toxin and further confirmed by cloning the gene that encodes ErgTx (forthcoming).

In fact, the ErgTx published by Gurrola et al. [1], with the amino acid sequence: DRDSCVDKSRCAKYGYYQECQDCCKNAGHNGGTQMFFKCKAP, does not correspond to the physiological functions described. The amino acid sequence of the new isoform, reported here, is the one used for the experiments previously reported [1], whose amino acid sequence is: DRDSCVDKSRCAKYGYYQECQDCCKNAGHNGGTCMFFKCKCA (GenBank accession number CnErg1). This sequence was obtained by direct Edman degradation using a Beckman LF3000 Protein sequencer instrument, which allowed unequivocal identification of the first 41 amino acid residues. The alanine in position 42 was confirmed by mass spectrometry analysis, after carboxypeptidase-Y hydrolysis of reduced and alkylated toxin with iodoacetamide, following a method previously described [5]. The molecular mass of this new ErgTx is 4730.8 ± 0.4 Da (theoretical value 4730.4 Da), which after reduction and alkylation gave the mass of 5195 Da (compatible with the presence of eight

cysteines), hence confirming unequivocally the presence of four disulfide bridges in the native ErgTx. The published sequence (first one listed above) is thought to be another isoform, which is now under investigation. This apparent confusion came from the usage of different chromatographic procedures to obtain peptides active on ERG-channels (paper in preparation, to be published elsewhere). All the physiological work performed and included in our initial reference [1] was done with the same batch of peptide, and the same sample was used for sequencing and disulfide bridge determination reported here.

Native toxin was digested with trypsin at pH 6.3 in order to avoid scrambling phenomena and the resulting peptide mixture was directly loaded, either in a MALDI-TOF DE-PRO (PerSeptive Biosystems) or in a Platform single quadrupole mass spectrometer (Micromass) and analyzed [3,4]. The spectra obtained are shown in Fig. 1. In addition to ErgTx, four clear signals were assigned to S–S bridged peptides on the basis of their unique mass values and their disappearance following incubation with dithiothreitol. The signal at *m/z* 1703.0 was interpreted as arising from peptides (11–13) and (26–38) linked by a disulfide bond between Cys-11 and Cys-34; its satellite peak at *m/z* 1718.8 was associated to the same peptide presenting Met-35 in oxidized form. Finally, the intense MH^+ signals at *m/z* 2858.2 and 2876.4 were associated to a three- and four-peptide clusters involving fragments (1–8)+(14–25)+(39–42) and (1–8)+(14–25)+(39–40)+(41–42) respectively, linked by three disulfide bonds between Cys-5, Cys-20, Cys-23, Cys-24, Cys-39 and Cys-41. These findings were confirmed by MALDI-MS analysis of the entire digest following treatment with carboxypeptidase B and/or sequence analysis of the corresponding peptide fractions obtained from HPLC purification. The assignment of other cysteine pairings was obtained by a consecutive digestion of this digest with endopeptidase AspN. In addition to the signal associated to the peptide (11–13)+(26–38) (not containing Asp residues), the spectrum showed five clear peaks at *m/z* 1301.5, 1321.9, 2343.6, 2361.9 and 2379.7. All signals disappeared following addition of dithiothreitol. The former was interpreted as arising from peptides (14–21)+(39,40) linked by a disulfide bridge between Cys-20 and Cys-39. The second one was assigned to its complementary three-peptide cluster involving fragments (3–8), (22–25) and (41–42) linked by the two possible disulfide bonds Cys5–Cys23 and Cys24–Cys41 or Cys5–Cys24 and Cys23–Cys41. On the basis of the sequence homology, secondary element conservation and the identical arrangement of two of the four disulfides with respect to other known toxins [2], we concluded that the cysteine pairing in this new ErgTx isoform is Cys5–Cys23, Cys11–Cys34, Cys20–Cys39 and Cys24–Cys41. Therefore, the remaining signals in the spectrum were associated to the three-, four- and five-peptide clusters involving fragments (3–6)+(14–25)+(39–42), (3–6)+(14–21)+(22–25)+(39–42) and (3–6)+(14–21)+(22–25)+(39–40)+(41,42) respectively, linked by the disulfide bonds Cys5–Cys23, Cys20–Cys39 and Cys24–Cys41.

Thus, the disulfide bridges of ErgTx are formed between cysteines: Cys5–Cys23, Cys11–Cys34, Cys20–Cys24 and Cys23–Cys41.

Acknowledgements: This work was supported in part by grants from Howard Hughes Medical Institute (75197-527107), National Council of Science and Technology Mexico (CONACyT-31691-N) and Dirección General del Personal Académico-UNAM (IN-217997) to L.D.P.

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*Corresponding author. Fax: (52)-73-172388.
E-mail: possani@ibt.unam.mx

[a] *I.A.B.B.A.M. Centro Internazionale Servizi di Spettrometia di Massa, CNR, Naples 80147 Italy*
[b] *Department of Molecular Recognition and Structural Biology, Institute of Biotechnology, National Autonomous University of Mexico, Avenida Universidad 2001, P.O. Box 510-3, Cuernavaca 62210 Mexico*
[c] *Department of Biotechnology and Biosciences, University of Milano–Bicocca, Milan 20126 Italy*