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Mini review

New insights into the membrane-binding and activation mechanism of pyruvate oxidase from *Escherichia coli*

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ABSTRACT

Pyruvate oxidase from *Escherichia coli* (*Ec*POX) is a thiamin diphosphate- (ThDP) and FAD-dependent peripheral membrane protein that carries out the irreversible oxidative decarboxylation of pyruvate to acetate and carbon dioxide. Concomitant two-electron reduction of the flavin cofactor was suggested to induce a structural rearrangement of the C-terminus triggering recruitment of the protein from the cytosol to the cell membrane, where the electrons are eventually transferred to final electron acceptor ubiquinone 8. Binding to the membrane, or alternatively, mild proteolytic digestion leads to a multifold enhancement of both the catalytic activity and substrate affinity. Recent X-ray crystallographic studies on *Ec*POX in the resting state and on a C-terminal truncation variant mimicking the membrane-bound activated form have fueled our understanding of the membrane-binding mechanism and concomitant catalytic activation. In the resting state, the auto-inhibitory C-terminal membrane anchor adopts a half-barrel/helix fold that occludes the active site. Upon activation, the C-terminus is expelled and becomes structurally flexible thereby freeing the active site. Circular dichroism spectroscopic analysis revealed the isolated C-terminus to be disordered, however, formation of a helical structure was observed in the presence of micelles. Limited proteolysis experiments indicate that activation of *Ec*POX involves at least two sequential structural transitions: the first occurring after binding of pyruvate to ThDP and the second after two-electron reduction of the flavin.

1. Introduction

The homotetrameric flavoenzyme pyruvate oxidase from *Escherichia coli* (*Ec*POX, EC 1.2.2.2) catalyzes the oxidative decarboxylation of pyruvate to acetate and CO_2 and requires the cofactors ThDP, Mg^{2+} and FAD for catalytic activity [1]. Unlike in the related POXs from *Lactobacillae* or pyruvate dehydrogenase multienzyme complex (PDHc), oxidation of pyruvate is not coupled to formation of an energy-rich metabolite such as acetyl phosphate (POX) or acetyl-CoA (PDHc), but is just exploited to feed two reducing equivalents from the cytosol into the respiratory chain at the membrane [2–5]. Therefore, *Ec*POX has been sometimes considered to be non-essential for *E. coli* metabolism serving as a seemingly wasteful backup system to PDHc. However, growth studies on *E. coli* *Ec*POX null mutants revealed a significantly reduced growth rate clearly suggesting an essential role of *Ec*POX during aerobic growth [6].

In the resting (non-activated) state, *Ec*POX localizes to the cytosol and exhibits a low basal catalytic activity ($k_{cat} \sim 5–10\,s^{-1}$) and poor affinity for its substrate pyruvate ($K_M^{app} \sim 90 - 100\,mM$) when monitored in reductase assays, which rely on reduction of externally added artificial electron acceptors such as ferricyanide or 2,6-dichlorophenolindophenol (DCPIP) [7]. The steady-state kinetic constants of the non-activated enzyme implicate a role for *Ec*POX at certain metabolic situations with elevated pyruvate concentrations.

The catalytic sequence of *Ec*POX involves as series of elementary catalytic steps and reaction intermediates consistent with the general Breslow mechanism of thiamin catalysis. After carbonyl addition of pyruvate to ThDP and decarboxylation of the resultant 2-lactyl-ThDP (LThDP) adduct, two reducing equivalents are transferred from the 2-(1-hydroxyethyl)-ThDP (HEThDP) carbanion/enamine intermediate to the neighboring flavin cofactor (Eq. (1)). Reduction of FAD has been suggested to induce a structural rearrangement of *Ec*POX that leads to the expulsion of a high affinity lipid binding site at the C-terminus [8–10]. Subsequently, *Ec*POX is recruited to the biological membrane and the reduced flavin transfers two electrons to the final electron acceptor ubiquinone 8

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(Q_8) that is dissolved within the membrane and serves as a mobile electron carrier of the electron transport chain (Eq. (2)).

$$\text{ThDP-}Ec\text{POX-FAD} + \text{CH}_3\text{–CO–CO}_2^- + \text{H}_2\text{O} \rightarrow \text{ThDP-}Ec\text{POX-FADH}_2 + \text{CO}_2 + \text{CH}_3\text{–CO}_2^- \quad (1)$$

$$\text{ThDP-}Ec\text{POX-FADH}_2 + \text{Q}_8 \rightarrow \text{ThDP-POX-FAD} + \text{Q}_8\text{H}_2 \quad (2)$$

Binding of reduced *Ec*POX to the membrane in vivo (or of lipid amphiphiles under in vitro conditions) leads to a few hundred-fold enhancement of the catalytic efficiency (k_{cat}/K_M^{app}) of *Ec*POX [8]. The activation is of a so called mixed *V*- and *K*-type as both turnover (V_{max} ~30-fold increased) and substrate affinity (K_M^{app} ~ 10-fold decreased) are affected. The same activation phenomenon is observed when reduced *Ec*POX (ThDP-*Ec*POX-$FADH_2$) is subjected to mild proteolytic digestion by α-chymotrypsin that cleaves off the last 23 C-terminal amino acid residues by clipping the peptide bond between residues Tyr549 and Met550 to give a 59 kDa fragment (*Ec*$POX_{\Delta 23}$) and a 23mer peptide, termed the 'alpha-peptide' [9,11,12]. On account of the similar kinetic steady-state constants of lipid/membrane activated enzyme on one hand and proteolytically activated material on the other, it has been deduced that both activation processes yield enzyme species with similar structural and functional properties. Therefore, the C-terminal Δ23 truncation variant is commonly deployed as a functional substitute for the lipid-activated enzyme.

There are multiple lines of evidence supporting a model in which membrane binding of *Ec*POX is conferred by the alpha-peptide part of the C-terminus. Cronan and co-workers reported that a genetically generated C-terminal Δ24 deletion variant of *Ec*POX is fully active even in the absence of lipids [13] paralleling the results obtained with the proteolytically activated enzyme, whereas bacterial strains expressing the deletion variant were lacking in vivo pyruvate oxidase activity. Furthermore, the truncation variant did not bind to detergents or lipids and no further increase of its activity could be observed in the presence of these potential activators. Mutagenesis studies indicated vital roles of several amino acid residues of the C-terminus such as Glu564 and Arg572 for in vivo oxidase activity of *Ec*POX and membrane binding [14]. A deficiency in lipid activation was also reported for variants with substitution in other domains (Ala467) [15], so it was concluded that activation and membrane binding require the C-terminal alpha-peptide to act in concert with neighboring structural elements. Disulfide cross-linking studies on different heterotetramers of *Ec*POX consisting of native subunits and subunits lacking the C-terminus showed that a pair of C-termini is the minimum requirement for membrane binding [16]. Sequence analysis of the alpha-peptide predicted it to fold into an amphipathic helical structure [17]. Conforming with this proposal, the isolated alpha-peptide was demonstrated to bind tightly to phospholipid vesicles in vitro [18].

Despite the extensive work on *Ec*POX, the structural basis of membrane binding and concomitant activation had remained elusive. Recent X-ray crystallographic structure analysis of full-length *Ec*POX and of the fully active, proteolytically activated *Ec*$POX_{\Delta 23}$ in combination with limited proteolysis studies, and structural studies on chemically synthesized alpha-peptide have now provided the structural framework for conceiving the underlying principles of membrane binding of *Ec*POX [19,20].

2. Results

2.1. Evidence for multiple conformational equilibria with relevance for catalysis and activation

Earlier studies using limited proteolysis revealed that *Ec*POX undergoes a major conformational transition in the course of catalysis [9]. In the absence of the substrate pyruvate, treatment of *Ec*POX with α-chymotrypsin results in the cleavage of a 101mer peptide (referred to as the 'beta-peptide') from the C-terminus rendering the enzyme catalytically inactive. After reaction of *Ec*POX with pyruvate and two-electron reduction of the flavin, however, the latter cleavage site is no longer accessible and only the last C-terminal 23 amino acids (the 'alpha-peptide') are removed to yield the fully active Δ23 truncation variant. In order to further delineate the catalytic stage(s) at which the conformational reorganisation takes place, the proteolytic pattern of *Ec*POX at defined intermediate states (pre-decarboxylation, post-decarboxylation, post-redox) of the thiamin and flavin cofactors were studied (Fig. 1).

At first, *Ec*POX was reacted with methyl acetylphosphonate (an electrostatic analog of pyruvate), which forms a covalent bond with C2 of ThDP to give the stable pre-decarboxylation intermediate analog phosphono-LThDP [21]. Secondly, the apo-FAD enzyme was reconstituted with Mg^{2+} and the post-decarboxylation analog thiazolone thiamin diphosphate that mimics the HEThDP enamine [22]. Finally, the holoenzyme was reductively titrated with sodium dithionite under strict anaerobic conditions to generate the two-electron reduced flavin thus resembling the situation after electron transfer (post-redox) from the HEThDP enamine to FAD.

As opposed to the results obtained with *Ec*POX in the resting state (cleavage of beta-peptide) or in the presence of pyruvate (cleavage of alpha-peptide), no proteolytic processing at all was observed with either covalent intermediate analog at the thiamin

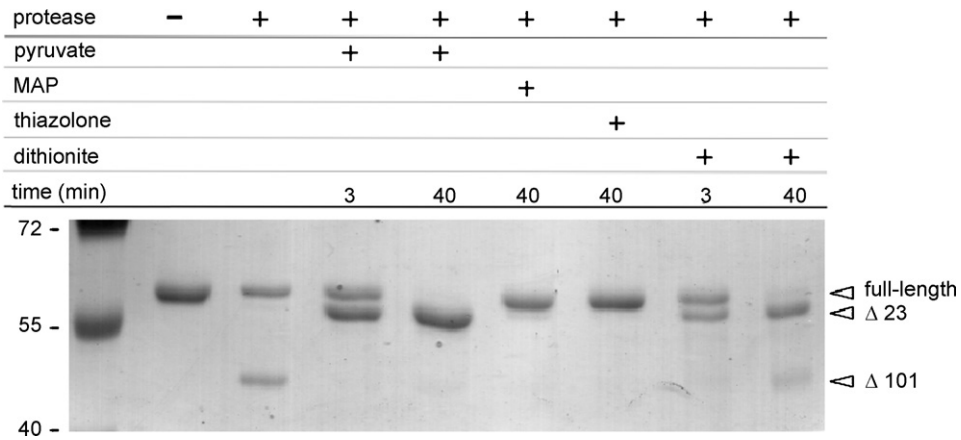

Fig. 1. SDS-page analysis of *Ec*POX after limited proteolytic digestion with alpha-chymotrypsin at different catalytic stages: (a) in the resting state, (b) after reaction with pyruvate, (c) after reaction with pyruvate analog methyl acetylphosphonate (MAP), (d) after reconstitution with HEThDP enamine analog thiamin-thiazolone diphosphate and (e) after full reduction of enzyme-bound FAD by sodium dithionite under strict anaerobic conditions.

site demonstrating that both potential cleavage sites are protected. On the other hand, the 23mer alpha-peptide is cleaved after full reduction of the enzyme-bound flavin. This indicates that there are two structural transitions in the course of catalysis, the first of which is caused by the formation of covalent intermediates at the thiamin site and the second by reduction of the flavin. The latter step is necessary for exposing the lipid binding site, suggesting a redox-sensing mechanism.

2.2. Structure of full-length *Ec*POX and of the C-terminal membrane anchor

The structure of full-length *Ec*POX was determined by X-ray crystallography to 2.9 Å resolution. Two functional dimers form the homotetramer as the biologically relevant unit. The four identical active sites are located at the dimer interface as reported for most enzymes of the ThDP enzyme superfamily [23]. Each subunit of *Ec*POX consists of three main domains typical for the pyruvate oxidase subfamily: a Pyr domain (residues 1–182), an FAD-binding domain (183–344) and a PP-binding domain (345–530). In addition, the membrane-binding C-terminus constitutes a small separate domain (531–572). The structural architecture of the tetramer implies that a pair of C-termini from neighboring subunits, each of which belonging to a different functional dimer, are likely to bind simultaneously to the membrane (Fig. 2A).

The C-terminal membrane-binding domain exhibits well-defined electron density for all residues and consists of two subdomains: a linker region (residues 531–549) and the alpha-peptide part (550–572) (Fig. 2B). The linker is composed of a single stranded β-sheet (531–534) and an α-helix (535–544), whereas the alpha-peptide part forms an antiparallel two-stranded β-sheet structure. The latter motif is part of a four-stranded half-barrel motif that – conjointly with the linker helix – partially occludes the substrate channel thereby impairing access to the active center. The last eight residues of the alpha-peptide part do not fold into a defined secondary structure. The C-terminal domain is firmly held in place by several intraloop hydrogen bonds and numerous hydrogen-bonding and electrostatic interactions formed between basic residues of the alpha-peptide part and aspartate side chains of a neighboring, partially unwound helix of the FAD domain. In addition, the side chains of Glu564 located at the alpha-peptide part and Gln537 of the linker helix form a hydrogen bond. In summary, the X-ray structure reveals that the C-terminus is tightly clamped to the protein surface and blocks the funnel that provides access to the active site.

2.3. Circular dichroism spectroscopic analysis of chemically synthesized alpha-peptide

Primary structure analysis initially suggested the membrane anchoring alpha-peptide to fold into an amphipathic helix involving residues Gly559-Asp-Glu-Val-Ile-Glu-Leu-Ala-Lys-Thr568 as important elements of the presumed helical structure [17]. In the resting non-activated state, *Ec*POX exhibits no affinity for phospholipids and does not bind to membranes. The above detailed structural analysis of full-length *Ec*POX showed that the alpha-peptide is part of a half-barrel super-secondary structure and forms multiple intra- and inter-domain contacts. In order to experimentally test the predicted helix propensity, we chemically synthesized the 23mer alpha-peptide and subjected it to circular dichroism (CD) spectroscopic analysis (Fig. 3). Far-UV CD spectra of the alpha-peptide in potassium phosphate buffer indicate the alpha-peptide to be mostly unstructured/disordered with negligible amounts of β-sheet and turn elements thus clearly ruling out an intrinsic sequence-encoded helix propensity of the alpha-peptide. However, in the presence of SDS micelles, which serve as a very simple mem-

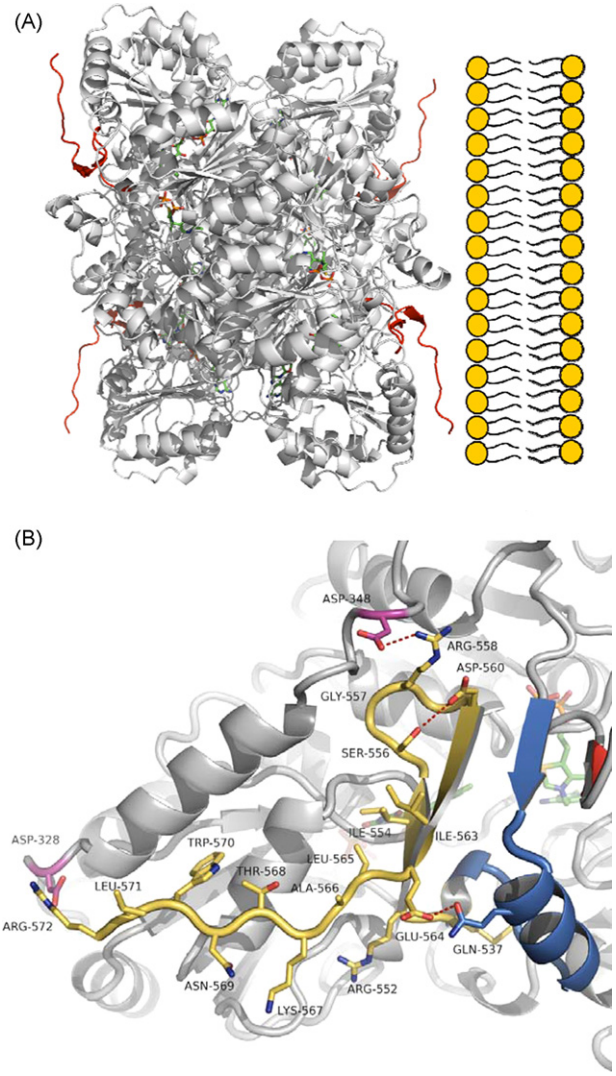

Fig. 2. Structure of the C-terminal membrane-binding domain of *Ec*POX. (A) The structure of the *Ec*POX homotetramer suggests that a pair of C-terminal alpha-peptide parts (res. 550–572, indicated in red) binds simultaneously to the biological membrane. (B) Cartoon representation of the C-terminal domain of *Ec*POX highlighting the linker region (res. 531–549, in blue) and the alpha-peptide (res. 550–572, in yellow). Hydrogen-bonding and electrostatic interactions are indicated. The two cofactors (in green) and selected amino acid side chains are shown in stick representation.

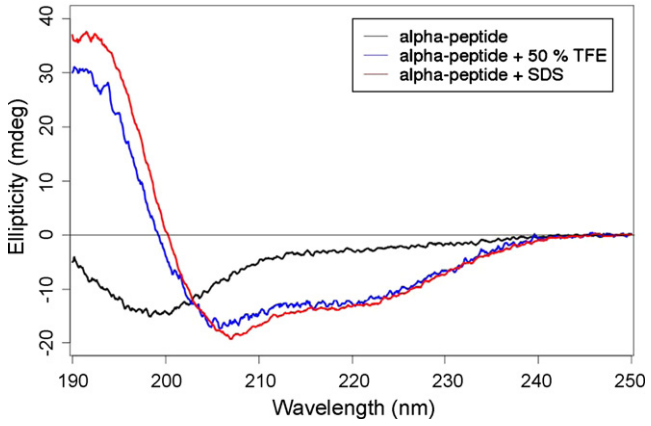

Fig. 3. Far-UV CD spectra of chemically synthesized alpha-peptide in buffer (black line), in presence of SDS micelles (red line) and in presence of 50% trifluorethanol (blue line).

brane mimic, the alpha-peptide adopts a helical structure as also observed with a positive control in presence of the helix-inducing compound trifluorethanol. From this experimental observation it can be concluded that activation and membrane binding of *Ec*POX involve at least two sequential structural transitions. At first, reduction of the flavin causes a local structural rearrangement that frees the C-terminus from its tight interactions with other domains and induces an order (half-barrel) – disorder transition. Formation of the presumed amphipathic helix will only occur upon contact of the C-terminus with the biological membrane.

2.4. Structural differences between the full-length and proteolytically activated EcPOX

The structural comparison of full-length *Ec*POX and the proteolytically generated C-terminal truncation variant *Ec*POX$_{\Delta 23}$ revealed that the conformational changes occurring in the course of activation are largely confined to the C-terminal segment and neighboring helices of the FAD domain (Fig. 4A). The α-helix encompassing residues 330–349 is now fully wound, whereas there is a slight structural displacement detectable for the adjacent helix $\alpha_{257-265}$, which in the full-length form is contacting the alpha-peptide part. Most intriguingly, the linker region of the C-terminus points away from the active site in *Ec*POX$_{\Delta 23}$ and access to the funnel leading to the active site is no longer impaired. The linker exhibits defined electron density only until Lys539 suggesting that the remaining part of the truncated C-terminus (residues 540–549) is highly flexible.

Another fascinating observation is the different structural orientation of an active center loop (encompassing residues 460–480) in the full-length enzyme and the truncated variant. In the full-length form, some residues of this loop form a β-sheet structure as part of the half-barrel occluding the active site. In the proteolytically activated enzyme, this loop swings deeper into the active center pocket contacting the thiamin and flavin cofactors. Most remarkably, the side chain of Phe465 is now approaching both the thiazolium moiety of ThDP and the isoalloxazine part of FAD (Fig. 4B). It is tempting to speculate about a catalytic role of Phe465 for electron transfer between the thiamin and flavin cofactor in terms of a 'way station', because catalytic activation of *Ec*POX by lipid binding and by limited proteolytic digestion was shown to mostly result from a dramatic rate enhancement of intercofactor electron transfer [24]. In support of this mechanistic suggestion, the suspected Phe is conserved in pyruvate oxidases but is missing in related enzymes of the pyruvate oxidase family such as acetohydroxyacid synthase or glyoxylate carboligase [25–27], which catalyze non-redox reactions and contain the non-catalytic flavin cofactor as a vestigial remnant of evolution [28,29].

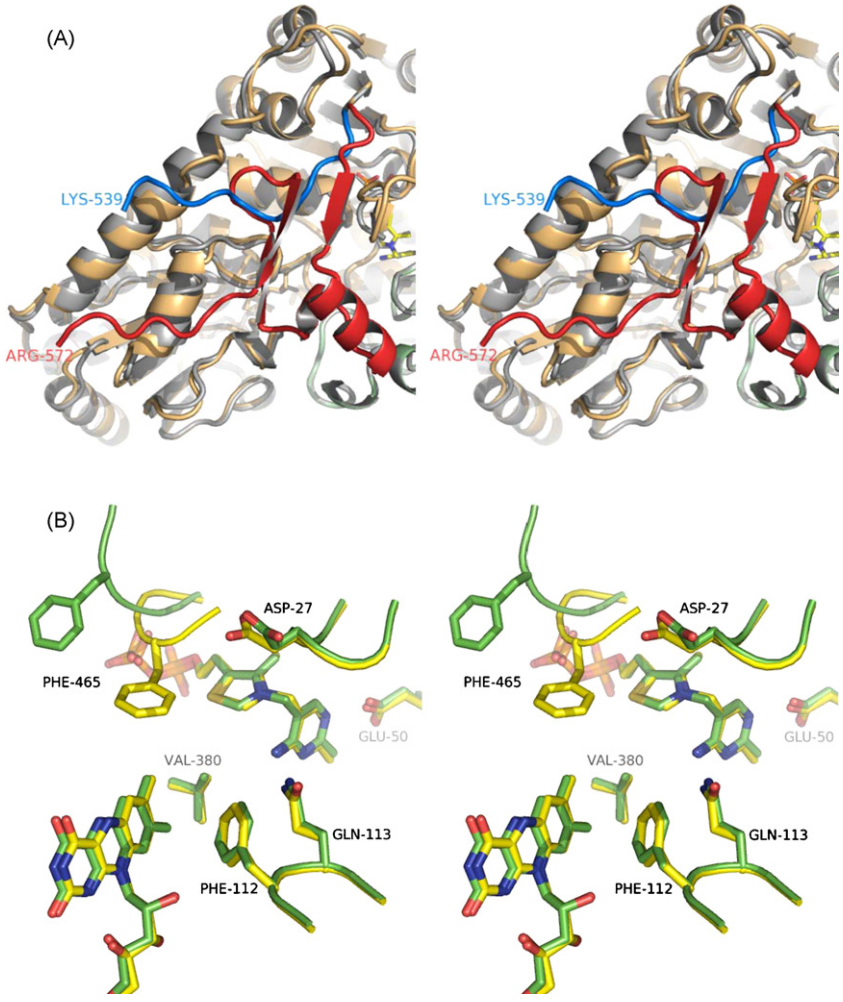

Fig. 4. Structural differences between full-length *Ec*POX and proteolytically generated *Ec*POX$_{\Delta 23}$ in stereo view. (A) Superposition of full-length *Ec*POX (monomers of the catalytic dimer coloured in orange and green) and *Ec*POX$_{\Delta 23}$ (both monomers coloured in gray). The C-terminal domains are highlighted: red, full-length *Ec*POX; blue, *Ec*POX$_{\Delta 23}$. (B) Superposition of the active sites of full-length *Ec*POX (in green) and *Ec*POX$_{\Delta 23}$ (in yellow) in stick representation.

3. Conclusions

Peripheral membrane proteins constitute an important class of proteins, which temporarily bind to the biological membrane and fulfill important functions in cellular regulation, signaling and metabolism [30,31]. In many cases, these proteins become recruited to the membrane after binding of effectors and substrates or after post-translational modification resulting in a conformational transition that drives membrane binding. *Ec*POX constitutes a very interesting example in this regard as it transfers reducing equivalents from the cytosol directly to the electron transport chain at the membrane. At 'normal' cellular pyruvate concentrations, *Ec*POX is located in the cytosol and adopts a structure in which the membrane-binding C-terminal domain is tightly clamped to the protein surface almost completely occluding the substrate channel. This explains the poor affinity of non-activated enzyme for its substrate pyruvate and its inability to bind to the membrane. At elevated cytosolic pyruvate concentrations, *Ec*POX will turn over pyruvate and the flavin cofactor will become reduced. Reduction of the flavin causes a structural rearrangement that eventually results in membrane binding of the C-terminus. The structural studies on a proteolytically activated Δ23 truncation variant of *Ec*POX and chemically synthesized membrane-binding peptide suggest that the C-terminus is expelled from the protein surface and becomes structurally flexible. Formation of the presumed amphipathic helix is likely to occur only upon contact of the C-terminus with the membrane. Release of the C-terminus from its interactions with other domains of the protein frees access to the active center. In addition, an active center loop is partially reorganized so that the side chain of Phe465 swings into the active center. In conjunction with transient kinetic studies, which evidenced a 10^2-fold rate enhancement of the intramolecular oxidation of the HEThDP enamine by the flavin, the structural data suggest a catalytic role of Phe465 for facilitating the redox reaction.

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References

[1] F.R. Williams, L.P. Hager, Journal of Biological Chemistry 236 (1961) PC36–37.
[2] L.P. Hager, F. Lipmann, Proceedings of the National Academy of Sciences of the United States of America 47, 1961, pp. 1768–1772.
[3] J.G. Koland, M.J. Miller, R.B. Gennis, Biochemistry 23 (1984) 445–453.
[4] K. Tittmann, G. Wille, R. Golbik, A. Weidner, S. Ghisla, G. Hübner, Biochemistry 44 (2005) 13291–13303.
[5] G. Wille, D. Meyer, A. Steinmetz, E. Hinze, R. Golbik, K. Tittmann, Nature Chemical Biology 2 (2006) 324–328.
[6] A.M. Abdel-Hamid, M.M. Attwood, J.R. Guest, Microbiology 147 (2001) 1483–1498.
[7] L.P. Hager, in: H. Bisswanger, J. Ullrich (Eds.), Biochemistry and Physiology of Thiamin Diphosphate Enzymes, 1991, pp. 277–285.
[8] C.C. Cunningham, L.P. Hager, Journal of Biological Chemistry 246 (1971) 1575–1582.
[9] M.A. Recny, L.P. Hager, Journal of Biological Chemistry 258 (1983) 5189–5195.
[10] M.W. Mather, R.B. Gennis, Journal of Biological Chemistry 260 (1985) 10395–10397.
[11] P. Russell, H.L. Schrock, R.B. Gennis, Journal of Biological Chemistry 252 (1977) 7883–7887.
[12] P. Russell, L.P. Hager, R.B. Gennis, Journal of Biological Chemistry 252 (1977) 7877–7882.
[13] C. Grabau, J.E. Cronan Jr., Biochemistry 25 (1986) 3748–3751.
[14] C. Grabau, Y.Y. Chang, J.E. Cronan Jr., Journal of Biological Chemistry 264 (1989) 12510–12519.
[15] Y.Y. Chang, J.E. Cronan Jr., Journal of Bacteriology 167 (1986) 312–318.
[16] Y.Y. Chang, J.E. Cronan Jr., Biochemistry 36 (1997) 11564–11573.
[17] M.A. Recny, C. Grabau, J.E. Cronan Jr., L.P. Hager, Journal of Biological Chemistry 260 (1985) 14287–14291.
[18] T.F. Zhang, L.P. Hager, Archives of Biochemistry & Biophysics 255 (1987) 201–204.
[19] A. Weidner, P. Neumann, G. Wille, M.T. Stubbs, K. Tittmann, Acta Crystallographica Section F 64 (2008) 179–181.
[20] P. Neumann, A. Weidner, A. Pech, M.T. Stubbs, K. Tittmann, Proceedings of the National Academy of Sciences of the United States of America 105, 2008, pp. 17390–17395.
[21] R. Kluger, D.C. Pike, Journal of the American Chemical Society 99 (1977) 4504–4506.
[22] J.A. Gutowski, G.E. Lienhard, Journal of Biological Chemistry 251 (1976) 2863–2866.
[23] Y.A. Muller, Y. Lindqvist, W. Furey, G.E. Schulz, F. Jordan, G. Schneider, Structure 1 (1993) 95–103.
[24] B.L. Bertagnolli, L.P. Hager, Journal of Biological Chemistry 266 (1991) 10168–10173.
[25] Y.A. Muller, G.E. Schulz, Science 259 (1993) 965–967.
[26] S.S. Pang, R.G. Duggleby, L.W. Guddat, Journal of Molecular Biology 317 (2002) 249–262.
[27] A. Kaplun, E. Binshtein, M. Vyazmensky, A. Steinmetz, Z. Barak, D.M. Chipman, K. Tittmann, B. Shaanan, Nature Chemical Biology 4 (2008) 113–118.
[28] Y.Y. Chang, J.E. Cronan Jr., Journal of Bacteriology 170 (1988) 3937–3945.
[29] K. Tittmann, K. Schröder, R. Golbik, J. McCourt, A. Kaplun, R.G. Duggleby, Z. Barak, D.M. Chipman, G. Hübner, Biochemistry 43 (2004) 8652–8661.
[30] W. Cho, R.V. Stahelin, Annual Review of Biophysics & Biomolecular Structure 34 (2005) 119–151.
[31] R.B. Cornell, S.G. Taneva, Current Protein & Peptide Science 7 (2006) 539–552.