Hypothesis

On the geometry of leukocyte NADPH-oxidase, a membrane flavoenzyme

Inferences from the structure of glutathione reductase

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Using the structure of glutathione reductase as a model, we suggest the following topography for leukocyte NADPH-oxidase: The binding sites of NADPH and O_2 are separated from each other by the flavin ring and are thus exposed to opposite sides of the plasma membrane. This model supports the concept that O_2^- is formed at the membrane facing the extracellular or phagosomal space, respectively. The fate of the proton produced in the reaction NADPH + 2 O_2 ⟶ NADP + 2 O_2^- + H^+ is also discussed in light of our model. NAD(P)H-oxidases possessing the topography of glutathione reductase may establish transmembrane proton gradients. Consequently our model suggests that leukocyte NADPH-oxidase produces not only the O_2^- burst but also a proton burst.

Leukocyte *Respiratory burst* *Cytotoxicity* *Membrane enzyme* *NADH,NADPH oxidoreductases* *Proton gradient*

1. INTRODUCTION

The oxidative mechanisms by which the neutrophilic leukocyte attacks microorganisms and tumour cells or causes tissue injury have been reviewed in [1–6]. The key enzyme in this process is an NADPH-oxidase of M_r 150000 [6–8]. This protein is located in the plasma membrane and in the phagosomal membrane which forms by invagination of the plasma membrane [9,10].

It was established in [7,8] that NADPH-oxidase is an FAD enzyme. Consequently it may be fruitful for the functional analysis of NADPH-oxidase to compare it with glutathione reductase, a flavoenzyme for which geometry and stereochemistry of catalysis are known in atomic detail [11–13].

2. COMPARTMENTS OF NADPH-OXIDATION AND O_2-REDUCTION

The overall reactions catalysed by NADPH-oxidase and glutathione reductase are very similar:

$$\text{NADPH} + O_2 + O_2 + H^+ \xrightarrow{\text{NADPH-oxidase}} \text{NADPH}^+ + 2\,O_2^- + 2\,H^+$$

$$\text{NADPH} + \text{GSSG} + H^+ \xrightarrow{\text{glutathione reductase}} \text{NADP}^+ + 2\,\text{GS}^- + 2\,H^+ \; (= \text{NADP}^+ + 2\,\text{GSH at pH 7})$$

When trinitrobenzene sulfonate is present, glutathione reductase indeed reduces O_2 and other compounds instead of GSSG [14]. Furthermore, both NADPH-oxidase and glutathione reductase

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are susceptible to thiol reagents [8,10,15] in the presence of NADPH, and both enzymes are (auto)inactivated in NADPH-dependent processes [6,7,16]. By inference from the known structure of glutathione reductase (fig.1) we should like to suggest the topography sketched in fig.2 for NADPH-oxidase.

According to this model (fig.2) NADPH is oxidized at the cytosolic side of the membrane. The reducing equivalents move via the flavin ring to the O_2-binding site oriented to the extracellular compartment or to the phagosomal compartment, respectively. Here the half-reaction $2\,O_2 + 2\,e^- \longrightarrow 2\,O_2^-$ takes place; the resulting O_2^- then undergoes further reactions [1,2,6,17–19]. In this way the cytotoxic oxygen derivatives are formed at the scene of action, namely at the surface of target cells or foreign particles.

The orientation of NADPH-oxidase in fig.2 is similar to that proposed by Babior and coworkers [18]. These authors, however, favour an intermembrane localisation of the O_2-binding region. Their major finding supporting this hypothesis, namely the resistance against inactivation by trypsin, could have other explanations; glutathione reductase (a cytosolic enzyme) behaves similarly towards trypsin under the conditions in [18] (Untucht-Grau, unpublished). Parallel experiments on NADPH-oxidase and glutathione reductase using trypsin and *p*-hydroxymercury benzoate as probes [18] might clarify this point.

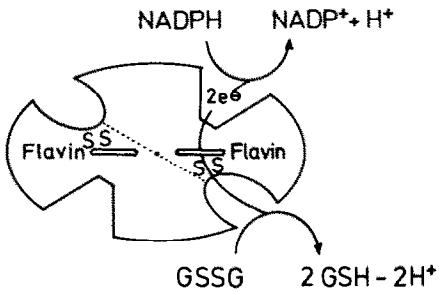

Fig.1. Topography of glutathione reductase [11–13]. The diad of the dimeric enzyme (bold dot in the centre) is oriented perpendicular to the paper plane. The dotted line represents the interface between the two subunits. A catalytic cycle is shown on the right-hand side. The electrons move from the nicotinamide of NADPH via the flavin ring and via the redox-active disulfide Cys-58/Cys-63 to glutathione disulfide. Regarding this isolated catalytic cycle a difference of $3\,H^+$ results between upper and lower surface of the enzyme. The difference amounts to $4\,H^+$ if $NADP^+$ is reduced again at its site of formation; e.g., by a dehydrogenase of the pentose phosphate cycle according to the equation

$$NADP^+ + \text{substrate} \cdot H_2 \longrightarrow H^+ + NADPH + \text{substrate}.$$

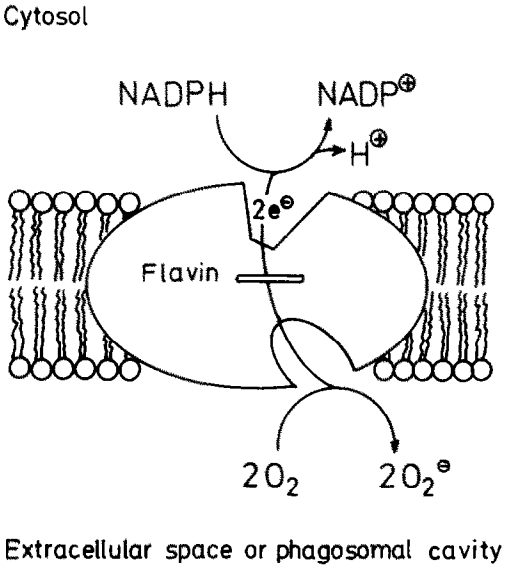

Fig.2. Proposed topography of leukocyte NADPH-oxidase. The model concerns both the site of proton release and the site of O_2-reduction. At the cytosolic side of the membrane the half reaction $NADPH \longrightarrow NADP^+ + H^+$ takes place; $2\,e^-$ are transferred to the external surface of the membrane where the half-reaction $2\,O_2 + 2\,e^- \longrightarrow 2\,O_2^-$ takes place. The possibility that NADPH-oxidase is a dimer with opposite polarities of the subunits [like glutathione reductase (fig.1)] would not change our conclusions since NADPH is available only on one side of the membrane. The postulated role of a *b*-type cytochrome [32] in the transfer of electrons from flavin to O_2 remains to be confirmed [6,7] and was, for clarity, not taken into account here.

3. COMPARTMENT OF H^+-PRODUCTION

The structure of glutathione reductase may be a helpful model for elucidating the (patho)physiological role of the protons which originate at a high rate during the respiratory burst (see balance equation for NADPH-oxidase and [20,21]). In the case of glutathione reductase (fig.1) there are probably $2\,e^-$ and no protons

transferred across the flavin ring [22,23]. As a consequence, a difference of 3 H^+ is created per catalytic cycle between opposite sides of the enzyme (fig.1). Since glutathione reductase is a cytosolic enzyme this gradient breaks down immediately due to proton transfer via the solvent.

Let us now consider the respiratory burst which is metabolically driven by the pentose phosphate cycle [1–6]. When $NADP^+$ is reduced in this cycle 1 H^+ is formed along with 1 NADPH in the cytosol. According to the model in fig.2, NADPH-oxidase releases a further H^+ (and $NADP^+$) at the cytosolic side of the membrane while 2 e^- travel to the external surface. This mechanism could contribute to the initial drop of pH in neutrophils exposed to chemotactic factors [20]. The protons released at the cytosolic membrane surface may have a role in the displacement of membrane-bound calcium thus triggering further events in the respiratory burst [5].

One should also test an alternative hypothesis concerning protons which is *not* shown in fig.2. As a flavoenzyme, NADPH-oxidase might transfer a hydride ion ($H^- \longrightarrow 2\,e^- + H^+$; [24,25]) or a hydride ion plus one proton ($H^- + H^+ \longrightarrow 2\,e^- + 2\,H^+$) to the external or phagosomal surface. These protons would, together with other mechanisms [26], contribute to the decrease in pH measured in phagosomes during the respiratory burst. The drop in pH in turn increases the concentration of $^{\cdot}O_2H$ ($pK = 4.6$) which is a much stronger oxidant than O_2^- itself [27]. In this context the results of van Zwieten et al. [21] should be recalled. They found a stoichiometric relationship between O_2-uptake and H^+-production during the respiratory burst. Their conclusion that NADPH-oxidase is not involved in this proton production appears premature to us since it was based on an experiment which did not consider all pitfalls [28] of determining O_2^- radicals. It should be stressed again that a concomitant translocation of electrons and protons is consistent with the overall topography but not with the detailed structure of glutathione reductase [22].

4. OUTLOOK

The geometry of glutathione reductase is present in other enzymes such as lipoamide dehydrogenase [29] and mercuric reductase [30] which facilitates comparative studies on these proteins. In the case of membrane flavoproteins which oxidize NADPH [1–6] or NADH [31] comparisons with glutathione reductase might help:

(i) To elucidate the translocation mechanisms of reducing equivalents;
(ii) To establish the role of H^+ which is probably released on one side of the membrane only.

As sketched in Fig.2, membrane NAD(P)H-oxidases possessing the glutathione reductase geometry can create transmembrane H^+-gradients.

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