Is cytochrome P-450$_{scc}$ a transmembrane protein?

Sergey A. Usanov, Alexey A. Chernogolov and Vadim L. Chashchin

Protein Chemistry Laboratory, Institute of Bioorganic Chemistry, BSSR Academy of Sciences, Minsk, USSR

Received 30 January 1990

The topology of cytochrome P-450$_{scc}$ in the inner mitochondrial membrane of adrenal cortex has been investigated using monospecific antibodies to cytochrome P-450$_{scc}$ and its fragments F_1 (Ile^1-Arg^{250}), F_2 (Asn^{257}-Ala^{481}) and F_3 (Asn^{257}-Arg^{399}). Antibodies to F_1 and F_2 were shown to effectively bind to the matrix and cytosolic sides of the inner membrane. Antibodies to F_3 specifically interacted only with the matrix side of the membrane. These data are consistent with a model of molecular organization which shows that cytochrome P-450$_{scc}$ is a transmembrane protein, both N- and C-terminal sequences of the cytochrome being able to span the membrane.

Cytochrome P-450$_{scc}$; Fragment; Monospecific antibody; Membrane topology; Bovine adrenal cortex

1. INTRODUCTION

Cytochrome P-450$_{scc}$ is the terminal component of cholesterol side chain cleavage system of adrenal cortex mitochondria. This system contains also a flavoprotein (adrenodoxin reductase) and an iron-sulfur protein (adrenodoxin). These proteins are thought to be localized in the inner mitochondrial membrane [1-3]. Adrenodoxin reductase and P-450$_{scc}$ seem to be typical integral proteins whereas adrenodoxin appears to be a peripheral one. The topology of the components of the mitochondrial monooxygenase system is unclear.

Recently, we succeeded in the preparation of monospecific antibodies to P-450$_{scc}$ fragments F_1 (Ile^1-Arg^{250}), F_2 (Asn^{257}-Ala^{481}) and F_3 (Asn^{257}-Arg^{399}) and showed that both N- and C-terminal sequences of P-450$_{scc}$ are involved in monooxygenation [4]. In the present work we used these antibodies to study the topology of P-450$_{scc}$ in the inner mitochondrial membrane of adrenal cortex.

2. MATERIALS AND METHODS

2.1. *Chemicals*

NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase were purchased from Boehringer (Mannheim, FRG). CNBr-activated Sepharose 4B and Protein A from *Streptococcus aureus* were obtained from Pharmacia (Uppsala, Sweden). Iodo-Gen was from Pierce (Rockford, IL, USA). Digitonin was from Merck (Darmstadt, FRG). Other chemicals were supplied by Serva (Heidelberg, FRG).

2.2. *Purification and preparation procedures*

P-450$_{scc}$, adrenodoxin and adrenodoxin reductase as well as affinity purified monospecific antibodies to P-450$_{scc}$ and its fragments F1, F2 and F3 were prepared as previously described [5]. Mitoplasts were prepared by treatment of bovine adrenal cortex mitochondria with digitonin [4]. The integrity of mitoplasts was proved by electron microscopy. To prepare sonicated mitoplasts, a suspension of mitoplasts (1–3 mg of protein/ml) was treated in a Virsonic cell disrupter 18-1850 (Virtis Co., Gardiner, NY, USA) (micro-tip, 30% of full intensity) twice for 1 min with a break for 2 min followed by the incubation for 20 min at 0°C.

2.3. *Enzymatic and chemical reduction of membrane-bound cytochrome P-450*

Mitoplasts were diluted with 50 mM phosphate buffer, pH 7.4, containing 0.3 M sucrose and 0.1 mM EDTA up to a protein concentration of 1.4 mg/ml and an aliquot was taken for sonication. To the sonicated and unsonicated samples adrenodoxin reductase and adrenodoxin were added up to 1.4 and 0.35 μM, respectively. The samples were saturated with CO and reaction was started by adding the NADPH-regenerating system [4]. When no further increase in the absorbance at 450 nm became observable, solid sodium dithionite was added to the sample and reference cells to chemically reduce the rest of cytochrome P-450.

2.4. *Interaction of antibodies with membrane-bound P-450$_{scc}$*

Mitoplasts in 0.2 ml (1.3 μg of protein) of 10 mM Tris-HCl buffer, pH 7.4, containing 0.3 M sucrose and 2% bovine serum albumin were incubated with increasing amounts of monospecific IgG for 2 h at 2°C. The mitoplasts were sedimentated by centrifugation for 20 min at 15 000 × *g*, washed with 1 ml of the same buffer and incubated with [^{125}I]Protein A (0.5 μg/tube) for 10 h at 2°C. Then the samples were centrifugated and pellets were washed twice with 1 ml of the same buffer. Radioactivity of the pellet was determined on a 1274 RIAGAMMA counter (LKB, Sweden). To determine non-specific binding of IgG with the membranes the same treatment of mitoplasts with the corresponding amounts of IgG from a non-immune rabbit was performed.

[^{125}I]Protein A with a specific radioactivity 2 mCi/mg was prepared by using Iodo-Gen as solid phase oxidizer. Mitoplast protein was determined by the method of Lowry et al. [6]. Spectral studies were conducted on a Specord M-40 (Carl Zeiss, GDR).

3. RESULTS

3.1. *Enzymatic reduction of membrane-bound cytochrome P-450*

Cytochrome P-450 (P-450$_{scc}$ + P-450$_{11\beta}$ in

Correspondence address: S.A. Usanov, Institute of Bioorganic Chemistry, BSSR Academy of Sciences, Zhodinskaya st., 5/2, Minsk, 220045, USSR

Abbreviations: P-450$_{scc}$ and P-450$_{11\beta}$, bovine adrenocortical cytochromes P-450$_{scc}$ and P-450$_{11b}$; F_1, F_2 and F_3, fragments of P-450$_{scc}$ polypeptide chain; PB, sodium phosphate buffer

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

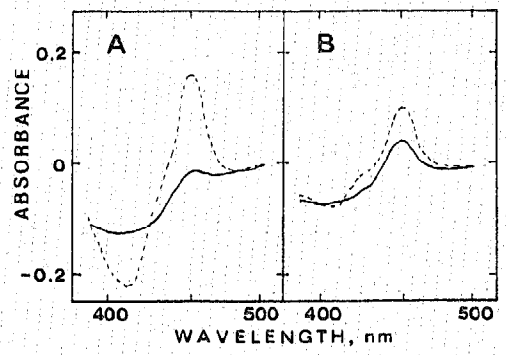

Fig. 1. Enzymatic (solid line) and chemical (dash line) reduction of membrane bound cytochrome P-450 in mitoplasts (A) and sonicated mitoplasts (B).

mitoplasts was not virtually reducible by NADPH neither in the absence (data not presented) nor in the presence (Fig. 1A) of exogenous adrenodoxin reductase and adrenodoxin. However, sodium dithionite was able to reduce cytochrome P-450 in mitoplasts as judged from the carbon monoxide difference spectrum of the reduced form of the hemeprotein. Sonication of mitoplasts, however, made cytochrome P-450 reducible by NADPH (Fig. 1B). The inability of exogenous adrenodoxin reductase and adrenodoxin to reduce $P\text{-}450_{scc}$ in mitoplasts indicates that neither the adrenodoxin reductase-binding site of adrenodoxin nor the adrenodoxin-binding site of $P\text{-}450_{scc}$ in mitoplasts are not exposed to the cytosolic side of the inner mitochondrial membrane.

3.2. *Interaction of antibodies to $P\text{-}450_{scc}$ and its fragments with membrane-bound hemeprotein*

To study the orientation of the polypeptide chain of $P\text{-}450_{scc}$ in the inner mitochondrial membrane, we used monospecific antibodies to assess the accessibility of membrane-bound $P\text{-}450_{scc}$ to these antibodies. Fig. 2A–C (lines 1) shows that incubation of antibodies to $P\text{-}450_{scc}$ and its fragments F1 and F2 resulted in their specific binding to the cytosolic side of the inner mitochondrial membrane as evidenced by binding of radioactivity to mitoplasts. The binding of antibodies to mitoplasts was highly dependent on the antibody/membrane protein ratio and increased with the increase of this ratio, saturation being reached at 30–40 weight excess of antibodies. The maximal binding took place with anti-$P\text{-}450_{scc}$ antibodies. However, antibodies to fragment F_3 representing the N-terminal portion of fragment F_2 was unable to specifically interact with the cytosolic side of the mitochondrial membrane (Fig. 2D) indicating that the sequence Asn^{257}-Arg^{399} is not accessible to antibodies in mitoplasts. This is in agreement with our recent finding that the polypeptide hinge connecting F_1 and F_2 is exposed to the matrix side of the inner membrane [4].

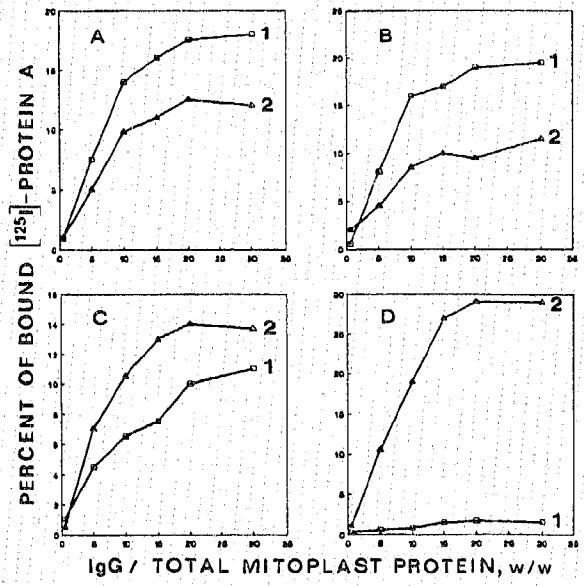

Fig. 2. Binding of monospecific antibodies against $P\text{-}450_{scc}$ (A), F_1 (B), F_2 (C) and F_3 (D) with mitoplasts (line 1) and sonicated mitoplasts (line 2). *y*-Axis represents radioactivity of pellets expressed as a percentage of total radioactivity added into each sample, the control radioactivity in the presence of the corresponding amounts of non-immune rabbit IgG (5–8.5%) being subtracted.

To investigate the antibody accessibility of the peptide segments corresponding to fragments F_1, F_2 and F_3 from the matrix side, binding of specific antibodies to sonicated mitoplasts was studied. Sonication of mitoplasts made $P\text{-}450_{scc}$ accessible to all the antibodies used (Fig. 2, lines 2). The binding of anti-$P\text{-}450_{scc}$ and anti-F_1 antibodies to sonicated mitoplasts was a little bit less compared to that to native mitoplasts. The binding of anti-F_2 and anti-F_3 antibodies to mitoplasts increased after sonication. In the latter case, the binding dramatically increased, indicating the appearance of sites from the matrix side of inner mitochondrial membrane which are accessible to specific interaction with antibodies against F_3.

4. DISCUSSION

In the present work we have shown the specific interaction of antibodies to $P\text{-}450_{scc}$ and fragments F_1 and F_2 (representing respectively the N- and C-terminal sequence of $P\text{-}450_{scc}$) with the cytosolic side of the inner mitochondrial membrane. These results are consistent with the view that $P\text{-}450_{scc}$ is a transmembrane protein, both N- and C-terminal segments being able to span the phospholipid bilayer (Fig. 3). The polypeptide hinge connecting both N- and C-terminal segments was previously shown to be exposed to the matrix side of the inner membrane [4]. The fact that antibodies to F_3 are not able to interact with the native mitoplasts further indicates that the sequence Trp^{400}-Ala^{481} of fragment F_2

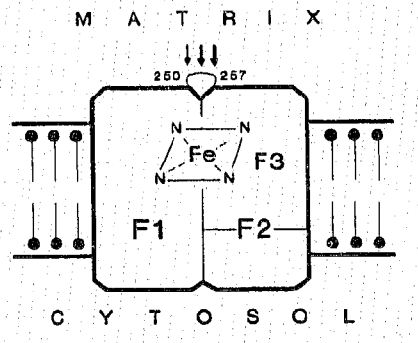

Fig. 3. Proposed model for the molecular organization of P-450$_{scc}$ in inner membrane of adrenal cortex mitochondria. Arrows show the hinge connecting F_1 and F_2 fragments which is accessible to trypsin.

of P-450$_{scc}$ is exposed to the cytosolic side of the inner membrane. Whether the C-terminal sequence of F_2 is really exposed to the cytosolic side of the inner mitochondrial membrane or that it spans the phospholipid membrane once more to return to the matrix side should be further clarified.

The fact that P-450$_{scc}$ in mitoplasts is not reducible by NADPH either in the absence or in the presence of exogenously added adrenodoxin reductase and adrenodoxin indicates that the adrenodoxin-binding site of P-450$_{scc}$ is exposed on the matrix side of the inner mitochondrial membrane. It was shown that lysine residues of both N- and C-terminal portions of P-450$_{scc}$ are involved in adrenodoxin binding [7]. This means that both N- and C-terminal segments should be exposed to the matrix side of the inner mitochondrial membrane. The increase of the binding of antibodies to fragment F_2 after sonication of mitoplasts provides further evidence that P-450$_{scc}$ polypeptide fragment Asn^{257}-Arg^{399} is exposed to the matrix side.

Recently, a model has been proposed which shows that cytochromes P-450 are bound to the endoplasmic reticulum membrane by only one or two transmembrane polypeptide segments at the N-terminus and that the active site and most of the polypeptide chain are exposed to the cytoplasm [8]. The data presented herein do not support such a model of membrane topology for the representative of ferredoxin-dependent cytochromes P-450 from bovine adrenocortical mitochondria. No evidence has been reported to support or reject such a model for membrane topology of P-450$_{scc}$. Therefore, the major question that remains to be answered is to clarify whether such topology is intrinsic for ferredoxin-dependent enzymes or only for P-450$_{scc}$.

REFERENCES

[1] Omura, T., Sanders, E., Estabrook, R.W., Cooper, D.Y. and Rosental, O. (1966) Arch. Biochem. Biophys. 117, 660–673.
[2] Churchill, P.F., de Alvare, L.R. and Kimura, T. (1978) J. Biol. Chem. 253, 4924–4929.
[3] Churchill, P.F. and Kimura, T. (1979) J. Biol. Chem. 20, 10443–10448.
[4] Usanov, S.A., Chernogolov, A.A. and Chashchin, V.L. (1989) FEBS Lett. 255, 125–128.
[5] Usanov, S.A., Chernogolov, A.A. and Chashchin, V.L. (1989) Biokhimiya (USSR) 54, 916–925.
[6] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J.Biol. Chem. 193, 265–275.
[7] Usanov, S.A., Chernogolov, A.A., Adamovich, T.B., Turko, I.V., Pikuleva, I.A. and Chashchin, V.L. (1989) in: Cytochrome P-450: Biochemistry and Biophysics (I. Shuster, eds) pp. 121–124, Taylor and Francis, London.
[8] de Lemos-Chiarandini, C., Frey, A.B., Sabatini, D.D. and Kreibich, G. (1987) J. Cell. Biol. 104, 209–219.