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Inhibitory domain-specific antibodies to cytochrome P-450$_{scc}$

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

Institute of Bioorganic Chemistry, BSSR Academy of Sciences, Zhodinskaya st., 5/2, Minsk, 220045, USSR

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Highly specific antibodies to cytochrome P-450$_{scc}$ and its F_1 and F_2 fragments, representing N- and C-terminal sequences of the hemeprotein respectively, were raised in rabbits. These antibodies were found to be inhibitory (up to 50–90%) for the cholesterol transformation into pregnenolone in the reconstituted system, indicating the involvement of both F_1 and F_2 domains formed by the respective fragments in monooxygenase catalysis. Cytochrome P-450$_{scc}$ in mitoplasts is not accessible for trypsin as revealed by immunological techniques. However, the treatment of submitochondrial particles with trypsin results in two main fragments identified by immunoblotting in the presence of the monospecific antibodies as F_1 and F_2 fragments. This indicates that the trypsin sensitive 250–257 region in cytochrome P-450$_{scc}$ molecule connecting both domains is exposed to the matrix side of the inner mitochondrial membrane.

Cytochrome P-450$_{scc}$ domain; Membrane organization; Monospecific antibody; Monooxygenase catalysis; Inhibition; (Bovine adrenal cortex)

1. INTRODUCTION

At least two cytochrome P-450-dependent systems are localized in the inner membrane of adrenocortical mitochondria: cholesterol side chain cleavage (scc) and 11b-hydroxylating ones [1]. Both P-450$_{scc}$ and P-450$_{11b}$ receive two electrons from NADPH via adrenodoxin reductase and adrenodoxin.

Limited proteolysis of the native purified P-450$_{scc}$ with trypsin results in two large fragments: F_1 with molecular weight of 29 800 Da and F_2 of 26 600 Da [2], corresponding to N- and C-terminal sequences of the hemeprotein, respectively (fig.1). These fragments appear to form domains differing in their functional properties [3,4]. The aim of the present work is to prepare monospecific antibodies to P-450$_{scc}$ and its structural fragments F_1, F_2 and F_3 (N-terminal sequence of F_2), and to use these antibodies in: (i) investigation of the role of structural elements in cholesterol conversion; (ii) study of P-450$_{scc}$ organization in membrane in comparison with purified protein.

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

Abbreviations: P-450$_{scc}$, bovine adrenocortical cytochrome P-450scc; F_1, F_2, F_3 and F_0, fragments of P-450$_{scc}$ polypeptide chain; PB, sodium phosphate buffer; SMP, submitochondrial particles; SDS-PAGE, SDS-electrophoresis in polyacrylamide gel

2. MATERIALS AND METHODS

2.1. Chemicals

NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase were purchased from Boehringer (Wien, Austria). [^{3}H]Cholesterol (58 Ci/mmol) was from Amersham International (Amersham, England). Complete Freund's adjuvant was from Calbiochem (La Jolla, CA, USA). Other chemicals were purchased from Serva (Heidelberg, FRG).

2.2. *Purification and proteolysis procedures*

P-450$_{scc}$, adrenodoxin and adrenodoxin reductase were isolated from bovine adrenocortical mitochondria as described [5]. Membrane-bound P-450$_{scc}$ preparates were isolated by treatment of the native mitochondria with digitonin (for preparation of mitoplasts) [4] following the sonication of mitoplasts (for SMP preparation) with subsequent washing of the SMP with 50 mM PB, pH 7.4, containing 0.2 M NaCl [6]. Proteolysis was done in the same buffer at the trypsin/P-450$_{scc}$ ratio 1:25. Then suspension of SMP was diluted 1:10 and centrifuged. The pellet was resuspended in 1 ml buffer and the supernatant was concentrated to the same volume for SDS-PAGE

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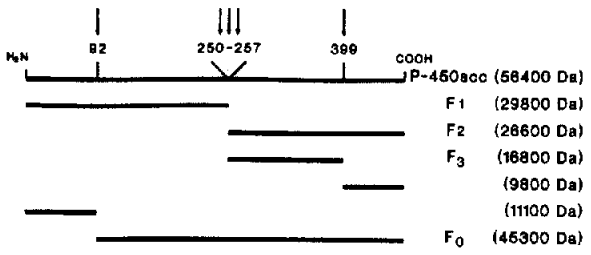

Fig.1. The main pathways of P-450$_{scc}$ trypsinolysis. Arrows show the sites in the P-450$_{scc}$ polypeptide chain accessible to trypsin.

samples preparation [4]. Trypsinolysis of purified P-450$_{scc}$ and separation of F_1, F_2 and F_3 were carried out as described in [2]. P-450$_{scc}$ concentration was determined as in [7].

2.3. *Immunological procedures*

100 μg of P-450$_{scc}$, F_2 or F_3 or 200 μg of F_1 in 0.35 ml of 50 mM PB, pH 7.4, containing 0.12% SDS, were mixed with equal volume of Freund's complete adjuvant and injected in several places in the neck of rabbits. This treatment was given intracutaneously during 6 weeks every other week. Blood samples taken from the marginal ear vein at 8 days after the last immunization were used for preparation of the antisera. Immunoblotting was performed as described in detail in [4].

2.4. *Assay of P-450$_{scc}$ activity*

The catalytic activity in reconstituted system in the presence of antibodies was examined as previously described [7]. P-450$_{scc}$ was preliminarily incubated with the antibodies for 10 min at 20°C. The reaction was initiated by addition of a NADPH-regenerating system containing 0.4 mM NADPH, 2.5 mM glucose-6-phosphate and 2 U/ml of glucose-6-phosphate dehydrogenase.

3. RESULTS

3.1. *Antigenic properties of F_1 and F_2*

Antibodies to P-450$_{scc}$ interact with hemeprotein, F_1, F_2 and F_3 as revealed by different immunological procedures including immunoblotting [4]. The finding that the structural elements of P-450$_{scc}$ retain antigenic properties became a prerequisite for further preparation of antibodies against these fragments. The antibodies obtained were shown to interact with P-450$_{scc}$ and corresponding antigens but they do not cross-react, as demonstrated by the same method (fig.2).

3.2. *Effect of the antibodies on P-450$_{scc}$-dependent cholesterol transformation*

Antibodies against P-450$_{scc}$ and its fragments proved to be potent inhibitors of the reaction

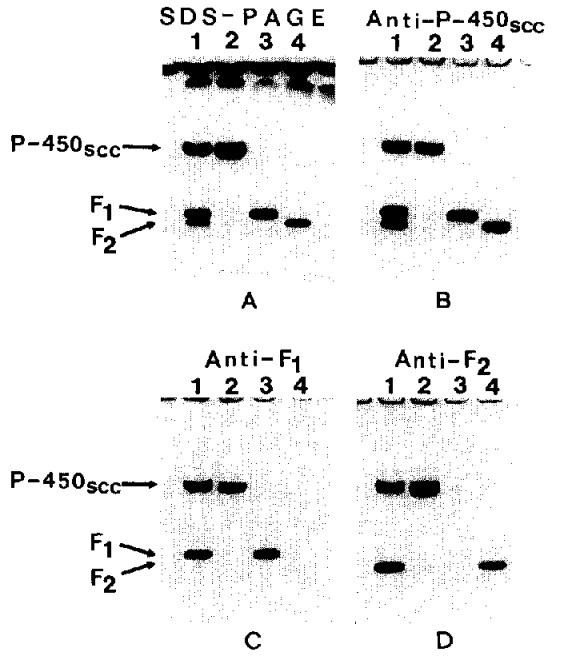

Fig.2. Immunoblotting of P-450$_{scc}$ and their fragments. (A) SDS-PAGE (12.5%) of the purified antigens. The samples contained: 1, P-450$_{scc}$ + F_1 + F_2; 2, P-450$_{scc}$; 3, F_1; 4, F_2. The gel was stained with Coomassie R-250. The blots B, C and D were treated with signed antisera diluted 1:200 and then these were stained by the peroxidase method [4].

catalyzed by the hemeprotein (fig.3). At the highest ratios of antibodies, the activity was inhibited by more than 80%. Generally, there was less inhibition of cholesterol metabolism by antibodies to F_2 and F_3 than when antibodies to F_1 were used. None of the antibodies used were able to inhibit cholesterol conversion into pregnenolone completely. The residual activity at the highest concentration of antibodies against P-450$_{scc}$ and its fragments might result from P-450$_{scc}$ molecules that are not accessible to antibodies since hemeprotein is in aggregated state [5].

3.3. *Proteolysis of membrane-bound P-450$_{scc}$*

To compare the molecular organization of purified and membrane-bound P-450$_{scc}$ we carried out the treatment of mitoplasts and SMP with trypsin. The products of trypsinolysis were detected by immunoblotting with antibodies against P-450$_{scc}$, F_1 and F_2. As follows from fig.4, membrane-bound P-450$_{scc}$ is subjected to limited proteolysis only in SMP. This conclusion is based on the fact

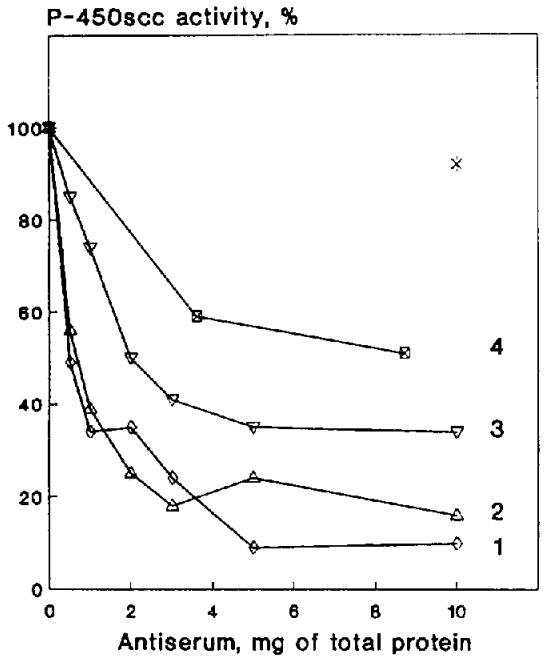

Fig.3. P-450$_{scc}$ activity in the presence of the antibodies. The samples contained increasing amounts of the following antisera: 1, anti-P-450$_{scc}$; 2, anti-F_1; 3, anti-F_2; 4, anti-F_3. The reconstituted system contained 1 nmol of P-450$_{scc}$, 1 nmol of adrenodoxin reductase and 5 nmol of adrenodoxin. Cholesterol concentration was 50 μM. The activity without any antiserum (20.1% of cholesterol transformation into pregnenolone) was taken as 100%. *, Activity in the presence of non-immune rabbit antiserum.

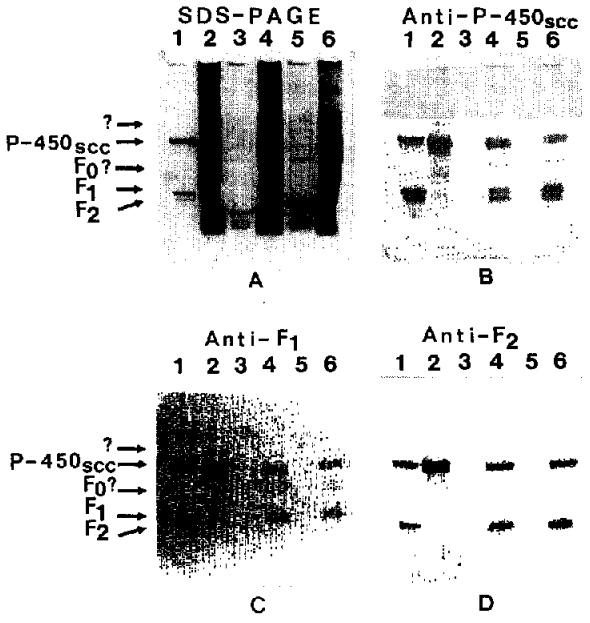

Fig.4. Trypsinolysis of membrane-bound P-450$_{scc}$. (A) SDS-PAGE (10%); (B, C and D) immunoblotting with signed antisera diluted 1:200. The samples (0.01 ml) contained: 1, purified P-450$_{scc}$ + trypsin (100:1, 20 min); 2, mitoplasts + trypsin (25:5:1, 80 min), supernatant + pellet; 3 and 4, SMP + trypsin (25:1, 20 min), supernatant and pellet, respectively; 5 and 6, SMP + trypsin (25:1, 80 min), supernatant and pellet.

that the antibodies recognize two main proteolytic products which are identical with F_1 and F_2 of purified P-450$_{scc}$ according to their molecular weights and immunological properties. The treatment does not result in elimination of any P-450$_{scc}$ products from membrane. It seems that region 250–257 in P-450$_{scc}$ molecule is exposed to matrix and both F_1 and F_2 are rigidly associated with membrane.

Moreover, some differences in proteolysis of purified and membrane-bound P-450$_{scc}$ appear to exist. In contrast to purified P-450$_{scc}$, during initial time of proteolysis of SMP a protein band with molecular mass of about 45 000 Da is recognized by all types of antibodies. This band appears to correspond to F_0 (fig.1) which is formed during trypsinolysis of pH-denatured or tetranitromethane-treated P-450$_{scc}$ [4]. Finally, we detected in untreated SMP an additional P-450$_{scc}$ band with higher molecular weight than native P-450$_{scc}$, which seems to correspond to a precursor of the hemeprotein [8].

4. DISCUSSION

Preparation of monospecific antibodies against functionally formed structural elements of P-450$_{scc}$ indicates that antigenic determinants are widely distributed in the polypeptide chain of the hemeprotein. This is a rather unique situation when polyclonal antibodies could be prepared against polypeptides carrying definite functional properties. The fact that antibodies against P-450$_{scc}$ and its fragments are inhibitors of cholesterol conversion into pregnenolone indicates that both P-450$_{scc}$ functional domains formed by F_1 and F_2 participate in monooxygenase catalysis.

Trypsinolysis of SMP confirms the similarity of the molecular organization of purified and membrane-bound P-450$_{scc}$. The results obtained in the present work also demonstrate that different

parts of P-450$_{scc}$ polypeptide chain are closely associated and interact during monooxygenase catalysis. This is in contrast to the simple model for P-450$_{scc}$ organization in phospholipid membrane according to which hemeprotein consists of two independent domains, a hydrophilic region that bears the heme group and a functionally distinct hydrophobic domain that anchors the molecule to membrane as in the cases of cytochrome b_5 [9] and cytochrome P-450 reductase [10].

REFERENCES

[1] Kimura, T. (1981) Mol. Cell. Biochem. 36, 105–122.
[2] Chashchin, V.L., Lapko, V.N., Adamovich, T.B., Lapko, A.G., Kuprina, N.S. and Akhrem, A.A. (1986) Biochim. Biophys. Acta 871, 217–223.
[3] Chashchin, V.L., Shkumatov, V.M., Usanov, S.A., Vasilevsky, V.I., Turko, I.V. and Akhrem, A.A. (1985) Biomed. Biochim. Acta 44, 665–677.
[4] Usanov, S.A., Chernogolov, A.A., Akhrem, A.A. and Chashchin, V.L. (1987) Biokhimiya (USSR) 52, 110–122.
[5] Chashchin, V.L., Turko, I.V., Akhrem, A.A. and Usanov, S.A. (1985) Biochim. Biophys. Acta 828, 313–324.
[6] Usanov, S.A., Chernogolov, A.A., Petrashin, A.I., Akhrem, A.A. and Chashchin, V.L. (1987) Biol. Membr. (USSR) 4, 1102–1116.
[7] Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2370–2378.
[8] Morohashi, K., Fujii-Kuriyama, Y., Okada, Y., Sogawa, K., Hirose, T., Inayama, S. and Omura, T. (1984) Proc. Natl. Acad. Sci. USA 81, 4647–4651.
[9] Dailey, H.A. and Strittmatter, P. (1978) J. Biol. Chem. 253, 8203–8209.
[10] Black, S.D. and Coon, M.J. (1982) J. Biol. Chem. 257, 5929–5938.