ISSN 0096-3925, Moscow University Biological Sciences Bulletin, 2011, Vol. 66, No. 2, pp. 65–67. © Allerton Press, Inc., 2011.
Original Russian Text © T.V. Ostroverkhova, R.V. Chertkova, A.N. Nekrasov, D.A. Dolgikh, M.P. Kirpichnikov, 2011, published in Vestnik Moskovskogo Universiteta. Biologiya, 2011, No. 2, pp. 000–000.

Design of Mutant Variants of Horse Cytochrome *C* by Analysis of Informational Structure Method and Testing its Biological Activity

T. V. Ostroverkhova[a], R. V. Chertkova[b], A. N. Nekrasov[b], D. A. Dolgikh[a], and M. P. Kirpichnikov[a]

[a] *Department of Bioengineering, Faculty of Biology, Moscow State University, Moscow, 119991 Russia*
e-mail: tato-tato@list.ru
[b] *Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow*

Received December 13, 2010

Abstract—Informational structure of cytochrome *c* was investigated using the ANIS method (Analysis of Informational Structure Method). Mutant variants of cytochrome *c* gene were constructed on the basis of data from the ANIS method. The mutations carry substitutions reducing electron-transport activity of cytochrome *c* in the mitochondrial respiratory chain. These mutant genes were obtained and expressed in the bacterial system. The biological activity of the obtained cytochrome *c* mutant variants interacting with complexes III and IV of the respiratory chain in the system of rat liver mitoplasts.

Keywords: cytochrome *c*, informational structure, ANIS method, respiratory chain, mitochondria
DOI: 10.3103/S0096392511020064

INTRODUCTION

The main function of cytochrome *c* is electron transport in the mitochondrial respiratory chain from complex III to complex IV. Accepting an electron from complex III, cytochrome *c* turns into the reduced form, while giving an electron to complex IV, it turns into the oxidized form. The oxidized and reduced horse cytochrome *c* forms differ in resonance chromophore raman spectra characterizing redistribution of electron density of heme porphyrin [1]. According to the protein informational theory, cytochrome *c* and hemoglobin may have a common mechanism of functioning due to the common cofactor. There are works devoted to the analysis of conformation of α and β hemoglobin subunits in different functional forms using the roentgen-structural analysis [2]. Change of hemoporphyrin ring stereochemistry of every subunit of hemoglobin when it comes from one form to another is characterized by iron atom shift relative to the macrocycle plane [2]. Cofactor restructuring related to the iron atom exit form the heme plane can be suggested to occur also in cytochrome *c* when an electron is transported in the respiratory chain.

According to the analysis of the cytochrome *c* informational structure, we suggested that the cytochrome *c* cofactor activity possibly related to the iron atom shift, the 76-83 amino acid sequence of cytochrome *c* is of principle significance. This area contains heme ligand Met-80 and, according to the calculated data, serves as a flexible connection for *N*- and *C*-terminal cytochrome *c* sequences providing flexibility of Met-80 and coordinated iron atom. To verify this suggestion on the basis of the ANIS method data, we constructed several mutant horse cytochrome *c* variants. Cytochrome *c* mutations were aimed to reduce the conformational flexibility of 76-83 amino acid sequence of the protein which must lead to the reduction of its electron transport activity.

MATERIALS AND METHODS

The ANIS method was used to investigate cytochrome *c* informational structure. Principal points of the method are shown on the ANIS-trees web server [3]. The introduction of the mutations in cytochrome *c* gene included in pBP(CYCS) plasmid vector was performed by site-directed mutagenesis QuikChange™ Mutagenesis Kit (Stratagene, United States). *Escherichia coli* JM109 strain was used for the expression of the obtained cytochrome *c* mutant genes. Cytochrome *c* mutant variants were isolated using ion-exchange and adsorption chromatography. Mitoplasts from rat liver mitochondria were obtained using Johnson and Lardy's method. Cytochrome *c* removal from liver mitochondria was performed by washing with a solution of high ionic strength [5]. Succinate and cytochrome *c* reductase activity of rat liver mitoplasts lacking cytochrome *c* was measured photometrically at 550 nm. The activities were expressed in μmole of reduced cytochrome *c* per min per mg of mitoplasts' protein. Cytochrome *c* oxidize activity of rat liver

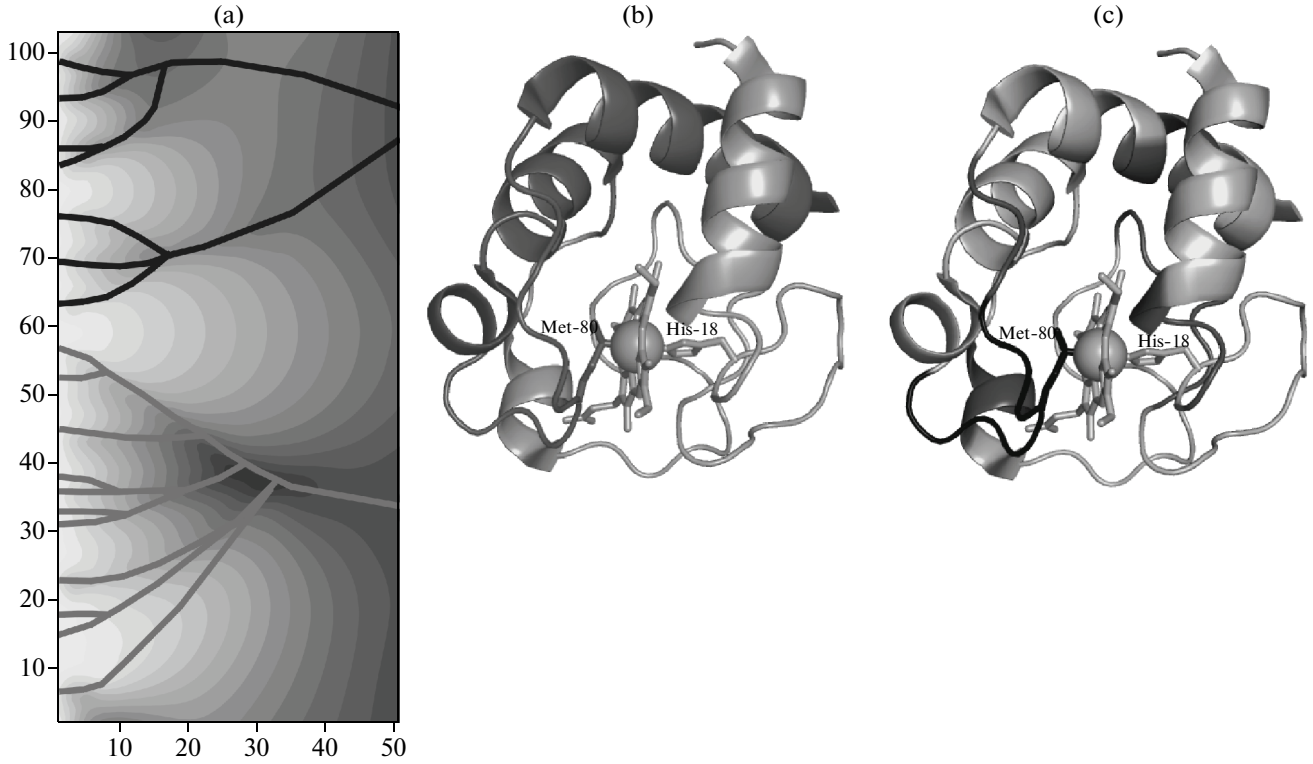

Fig. 1. A—Cytochrome *c* informational structure: X-axis—size of the site for which the function was calculated which describes the value of a.a. correlation; Y-axis—a.a. number in the cytochrome *c* sequence; Z-axis (tints of gray range)—value of function reflecting the degree of a.a. correlation; B—cytochrome *c* spatial structure where ELIS of the highest rank is highlighted; C—cytochrome *c* spatial structure where ADD-site is highlighted with black. Iron ligands His-18 and Met-80 are shown.

mitoplasts lacking cytochrome *c* was measured using an amperemeter with a closed platinum electrode. Activities were expressed in μmole of oxidated cytochrome *c* per min per mg of mitoplasts' protein [6].

RESULTS AND DISCUSSION

Investigation of the cytochrome *c* amino acid sequence by the ANIS method showed that the informational structure of cytochrome *c* is formed by two hierarchical elements of the informational structure (ELIS) of the highest rank: 1–58 a.a. and 59–104 a.a. (Fig. 1A) [3, 7]. Corresponding elements of the spatial organization are shown in Fig. 1B. In addition, one site with abnormally low density of ELIS (ADD-site) is marked out in the informational structure of cytochrome *c* (Fig. 1C) [3, 8]. The low density site is a part of one of two ELIS of the higher rank 59–104 a.a. and is interfacial between ELIS of the lower rank 59–79 a.a. and 80–104 a.a. The ADD-site contains the residuals 76–83, including iron ligand Met-80 (Fig. 1C). The second iron ligand His-18 is situated on the ELIS of the highest rank 1–58 a.a. Thus, the amino acid iron ligands are included in different ELIS of the highest rank which correspond to the extensive parts of cytochrome *c* polypeptide chain.

The informational structure of the other heme-containing protein hemoglobin with the amino acid ligands His-58 and His-87 is studied. The hemoglobin informational structure is shown to consist of two ELIS of the highest rank and each of these ELIS contains one amino acid iron ligand.

Therefore, the position of the iron ligands as functionally significant a.a. on different ELIS of the highest rank is typical for the informational structure of hemoglobin as well as cytochrome *c* and hydrolases [9]. In our opinion, iron atom shift accompanying the cofactor's work in hemoglobin is a result of movement of two big elements of the protein spatial structure corresponding to ELIS of the highest rank, each of which contains one iron amino acid ligand. Therefore, the heme ligands are located in different ELIS of the highest rank.

Presence of the mechanism analogous to hemoglobin and providing cofactor work in cytochrome *c* was assumed. The movement of ELIS of the highest and the lower rank relative to one another in cytochrome *c* can be provided by the flexibility of the 76–83 a.a. site (ADD-site) in the protein located interfacial between ELIS of the lower rank within one of ELIS of the highest rank. Thus, using a new theoretical idea about the cytochrome *c* structural organization, we proposed a model of functioning of this protein built with the ANIS method.

On the basis of the proposed model of cytochrome *c* functioning and calculated data obtained by the ANIS method, the cytochrome *c* mutant variants containing

substitutions in the ADD-site, I18Y/A83Y/G84N, T78N/K79Y/M80I/I81M/F82N, and T78S/K79P, were constructed. The mutations were directed to reduce protein ADD-site flexibility that, in our opinion, could reduce iron movements within heme and inhibit cytochrome *c* ability for electron transport in the respiratory chain. The mutant genes of cytochrome *c* were obtained and expressed in *E. coli* cells and the corresponding recombinant proteins were extracted in a preparative amount. To study the ability of the mutant cytochrome *c* variants to transport of electron, their interaction with the complexes III and IV was investigated. The highest decrease of the succinate:cytochrome *c* reductase activity of mitoplasts (for 97% from the activity in the presence of the wild type cytochrome *c*) was observed when the mutant variant T78S/K79P was added. The highest decrease of cytchrome *c* oxidase activity (for 85% from the activity in the presence of the wild type cytochrome *c*) was observed when the mutant variant I18Y/A83Y/G84N was added. The obtained data are similar to the results for the cytochrome *c* mutant variants constricted earlier [6] to eliminate electrostatic interactions of cytochrome *c* with the respiratory chain complexes. Possibly, a.a. substitutions in the N-terminal sequence of the single ADD-site are critical for cytochrome *c* interaction with complex III, while a.a. substitutions in the C-terminal sequence of the ADD-site are critical with complex IV.

Thus, the cytochrome *c* informational structure represented by two ELIS of the highest rank was investigated. Cytochrome *c* variants with the substitutions I18Y/A83Y/G84N, T78N/K79Y/M80I/I81M/F82N, and T78S/K79P directed to the decrease of its electron-transport activity was constructed. The mutant cytochrome *c* variants were obtained and investigated. The biological activity of the mutant variants was significantly reduced in comparison with the wild type cytochrome *c*.

ACKNOWLEDGMENTS

We would like to thank V.G. Grivennikova and A.D. Vinogradova (Department of Biochemistry, Faculty of Biology, Moscow State University) for assistance in cytochrome biological activity detection.

The study was supported by the Russian Academy of Sciences program Molecular and Cellular Biology.

BIOGRAPHIES

Tatyana Vladimirovna Ostroverkhova, PhD of Department of Bioengineering, Faculty of Biology, Moscow State University, Moscow, Russia, tel. 8(962)942-88-93; e-mail: tato-tato@list.ru

Rita Valer'evna Chertkova, Cand. Sci. (Biol.), Researcher, Laboratory of Protein Engineering, Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia, tel. 8(495)335-28-88; e-mail: rita@nmr.ru

Aleksey Norbertovich Nekrasov, Cand. Sci. (Phys.-Math.), Senior Researcher, Laboratory of Protein Biotechnology, Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia, tel. 8(495)335-43-66; e-mail: alexei_nekrasov@mail.ru

Dmitrii Aleksandrovich Dolgikh, Dr. Sci. (Biol.), Head of the Laboratory of Protein Engineering, Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Prof. of the Chair of Bioengineering, Faculty of Biology, Moscow State University, Moscow, Russia, tel. 8(495)335-28-88; e-mail: dolgikh@nmr.ru

Mikhail Petrovich Kirpichnikov, Dr. Sci. (Biol.), Academician, Russian Academy of Sciences, Dean of the Faculty of Biology, Head of the Department of Bioengineering, Faculty of Biology, Moscow State University, Head of the Department of Bioengineering, Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia, tel. 8(495)939-27-76; e-mail: kirpichnikov@inbox.ru

REFERENCES

1. Johnson, D. and Lardy, H., Isolation of Liver Or Kidney Mitochondria, *Methods Enzymol.,* 1967, vol. 10, pp. 94–96.
2. Jacobs, E. and Sanadi, D., The Reversible Removal of Cytochrome C from Mitochondria, *J. Biol. Chem.*, 1960, vol. 235, pp. 531–534.
3. Nekrasov, A., Petrovskaya, L., Toporova, V., Kryukova, E., Rodina, A., Moskaleva, E., and Kirpichnikov, M., Design of a Novel Interleukin-13 Antagonist from Analysis of Informational Structure, *Biokhimiya*, 2009, vol. 74, no. 4, pp. 493–500 [*Biochemistsry* (Moscow), 2009, vol. 74, no. 4, pp. 399–405].
4. Nekrasov, A. and Zinchenko, A., Structural Features of the Interfaces in Enzyme-Inhibitor Complexes, *J. Biomol. Struct. Dyn.*, 2010, vol. 28, no. 1, pp. 85–90.
5. Nekrasov, A. and Zinchenko, A., Hydrolases: The Correlation between Informational Structure and the Catalytic Centers Organization, *J. Biomol. Struct. Dyn.*, 2008, vol. 25, no. 5, pp. 553–562.
6. Pepelina, T., Chertkova, R., Ostroverkhova, T., Dolgikh, D., Kirpichnikov, M., Grivennikova, V., and Vinogradov, A., Site-Directed Mutagenesis of Cytochrome *c*: Reactions with Respiratory Chain Components and Superoxide Radical, *Biokhimiya*, 2009, vol. 74, no. 6, pp. 768–778 [*Biochemistsry* (Moscow), 2009, vol. 74, no. 6, pp. 625–632].
7. Paoli, M., Liddington, R., Tame, J., Wilkinson, A., and Dodson, G., Crystal Structure of T State Haemoglobin with Oxygen Bound at All Four Haems, *J. of Mol. Biol.*, 1996, vol. 256, no. (4), pp. 775–792.
8. Strekas, T. and Spiro, T., Cytochrome *c*: Resonance Raman Spectra, *Biochim. Biophys. Acta*, 1972, vol. 278, no. 1, pp. 188–192.
9. URL: http://anis.ibch.ru/trees/15.10.2010.

SPELL OK