

Catalytic centres of enzymes: structural paradoxes, the phenomenon of structural unity and new reactions

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DOI: 10.1070/MC2004v014n05ABEH002020

The large amount of data on enzyme structures obtained to date allow us to discuss general regularities in the formation of catalytic centres of enzymes. Approaches based on a comparison of structures in large data arrays made it possible to reveal a number of non-obvious regularities and to elicit principles of the design of catalytic centres.

In the past few decades, enzymes that have extraordinary catalytic efficiencies exceeding 10^{10} – 10^{15} times the efficiencies of classical chemical catalysts such as hydrogen or hydroxyl ions became the subject of a detailed study by modern physicochemical, structural and kinetic methods.

Intensive studies of enzymes as catalysts of chemical reactions resulted in a rather deep understanding of the physicochemical bases of enzyme catalysis and the origins of the high activity and specificity of enzymes.^{1–5}

The established concepts of molecular processes occurring in the active centres of enzymes within a catalytic cycle are based on the simultaneous use of two approaches. On the one hand, these are structural studies of enzymes as proteins based on data at atomic resolution; on the other hand, these are detailed kinetic studies of enzyme catalysis.^{6,7} The extremely ample structural and kinetic data obtained in the past decade now require systematization and generalization. This analysis that would allow general properties of enzyme catalysis as a natural phenomenon to be revealed at a molecular level may be based on the methods of bioinformatics.^{8–10}

The use of approaches and methods of bioinformatics allows one to discover certain non-obvious specific features of the structures of active centres.

Modern computer databases contain ample data on the structures of proteins and enzymes. This concerns both primary sequences of amino acids in polypeptide chains and structures of proteins at atomic resolutions.^{11–14} It is important that databases that allow large amounts of data to be handled also serve as a means for the systematization of structural data and for the analysis of such data by means of algorithms specific to each database.

Data on primary structures, that is, the sequences of amino acids in proteins, are most abundant. Studies on genomes gave huge amounts of data, which continue to increase exponentially.

It is well known that the primary sequence determines all

structural hierarchies of a protein. A scientific problem obviously arises: how can the full 3D structure of a protein, including the structure of its active centre, be forecasted based on a known sequence of amino acids? At present, at least two approaches are used to solve this problem. One is based on theoretical calculations of the energetics of a protein globule with a search for structures with minimum energies.¹⁵ This approach demands a large amount of computation. Furthermore, in the majority of cases, the problem does not have an unambiguous solution, though it can be expected that progress in this field will provide practically valuable results.

The second approach is considerably simpler: a structure is built by homology. This approach is based on the fact that many proteins, especially proteins from different sources, which perform the same function or similar functions, are very much alike and have a high degree of homology. Since full 3D structural information is available for many proteins, it is possible to use these structures to build the structure of a homologue. This simplifies the computation considerably.

To reveal the amino acids that form the active centre of an enzyme and the structure-forming amino acids that ‘assemble’ the side radicals of the amino acids to the catalytically active centre, we used an approach based on the comparison of amino acid sequences in homologous proteins belonging to a large family.^{8–10}

The procedure for comparing proteins that have a sufficiently high degree of homology (30% or higher) was called multiple alignment. If one protein in a set is taken as the base, other homologous proteins can be aligned to it by means of a computer-based procedure that compares the degree of identity of each position in the sequence within the entire set. The multiple alignment algorithm automatically takes into account the ‘insertions’ (additional sequences aside from the general sequence) and ‘deletions’ (missing partial fragments of the chain). Multiple alignment results in a matrix of probabilities of

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finding specific amino acids at each position of the polypeptide chain chosen as the base. This approach makes it possible to determine the positions of amino acids that have high probabilities of conservation of the given amino acid, that is, to find so-called conservative positions characteristic of all proteins in a set. In this case, the probability of finding the j th acid at the i th position is close to unity. On the other hand, this approach makes it possible to determine the positions which can contain virtually any of the twenty natural amino acids. A powerful filter that allows one to extract a 'signal' from statistical noise is provided by Shannon's entropy

$$H = -\sum_i p_i^j \log p_i^j,$$

where p_i^j is the probability or relative frequency of occurrence of the j th amino acid at the i th position.

A remarkable property of this function is that Shannon's entropy equals zero when the probability of the event equals unity, which allows the signal to be 'filtered off' from noise.

Data on the alignment of homologous proteins are presented in the HSSP database.¹³

We have applied the alignment procedure to homologous proteins: calculation of probabilities of finding the given amino acid at the given position and calculation of Shannon's entropy for each position in the sequence in order to determine the amino acids that constitute the active centre of the enzyme. The procedure allows one to identify with high reliability the active centre groups and structure-forming amino acids based on the knowledge of the sequence of amino acids in the protein polypeptide chain.^{5,9,10}

A necessary condition for the applicability of this approach is that the protein belongs to a sufficiently representative group of homologous proteins. The statistical sample should include at least 50 to 100 proteins. As an example, Figure 1 shows multiple alignment data and calculated Shannon's entropy values for Fe²⁺-dependent superoxidedismutases, *i.e.*, enzymes that eliminate superoxide radicals in biological systems.

Analysis of such dependences for a large number of enzymes makes it possible to reveal a number of interesting regularities. Some of the regularities discovered are non-obvious and paradoxical.

(1) The sequences of amino acids within a single protein family that has a well-defined biological function, *e.g.*, specific enzyme activity, are rather diverse. However, there are positions at which amino acids are virtually invariant; that is, the same particular amino acids remain at certain positions within the entire family (sometimes, up to 1000 proteins). These positions are found rather easily within the approach used since Shannon's entropy for such amino acids approaches zero at certain positions. Let us call them conservative positions and conservative amino acids, respectively.

(2) A large fraction of amino acids in a polypeptide chain of related enzymes is highly variable (Shannon's entropy varies in

the range from 1 to 3). During evolution and mutagenesis, nature has created a wide variety of related structures that have the same functions, *e.g.*, ability to perform an enzymatic reaction. The same enzyme can be isolated from different organisms (from *E.coli* to human beings). Such proteins differ considerably in composition and amino acid sequence but have a virtually identical structure of the catalytic centre. Note that the active centre of any enzyme is represented by two substructures: a binding centre, which ensures binding and orientation of the substrate relative to the catalytically active groups, and the catalytic centre itself, which performs the elementary acts of bonds redistribution. The wide variety of polypeptide chain sequences along with the virtually identical spatial structure of the catalytic centre shows that only a limited number of amino acids at certain positions of the polymeric chain are important when forming a catalytic centre. The natural variation of amino acids demonstrates that the majority of amino acids in a polypeptide chain can be replaced by other amino acids. Shannon's entropy is up to 2.5–3 at many positions, which suggests that virtually any of the 20 natural amino acids can occupy each of these positions. As a result, certain characteristics of proteins, such as stability, isoionic point, hydrophilicity or hydrophobicity, would change. However, the main function, *i.e.*, catalytic activity, and the structure of the catalytic centre remain invariant for the entire family.

(3) The amino acids forming a catalytically active centre always manifest themselves as conservative amino acids with Shannon's entropy approaching zero. This has been verified with a large set of enzymes with known structures of catalytic centres. The group of amino acids forming catalytic centres include aspartic acid, histidine, arginine, serine, and glutamic acid; that is, the amino acids that carry an expressed acid–base function capable of activating a molecule by the nucleophilic–electrophilic mechanism.

(4) Which amino acids appear most frequently as conservative? Amino acids were rated as conservative in terms of occurrence. Figure 2 shows this rating for the two largest classes of enzymes, *viz.*, hydrolases and oxidoreductases.¹⁶ It was found unexpectedly that glycine is the most essential amino acid, which is met most frequently as conservative. The paradox is that glycine, the simplest amino acid that has no expressed chemical function in the polypeptide chain, is the most essential amino acid in forming the protein structure and hence the active centre.

The second important place in the conservatism rating belongs to aspartic acid, which most commonly occurs in active centres as a nucleophile (in deprotonated form) or electrophile (in protonated form). Another extremely important amino acids include histidine with an imidazole group (for hydrolases) and arginine carrying a positively charged guanidine moiety (for oxidoreductases). Structure-forming amino acids, such as proline and cysteine, also rather commonly occur as conservative.

The distribution of functions of conservative amino acids of different types is rather obvious. The carboxy group of aspartic and glutamic acids, the imidazole group of histidine and the guanidine moiety of arginine form three-dimensional catalytic structures that perform concerted nucleophilic–electrophilic catalysis. The elementary act of catalysis involves a relatively small proton transfer (by 1–1.5 Å along the hydrogen bond) or polarization of the bond by an electrophilic agent. As a result, the reactivity of the substrate molecule or the active centre group increases by a factor of 10⁶–10⁷. For example, the transfer of a proton away from water, which approximates the structure of the reagent in the transition state to a hydroxyl ion, increases its reactivity in nucleophilic substitution by a factor of 10⁷. If the process occurs in the concerted mode and each substrate is activated in this way, acceleration of the reaction by a factor of 10¹²–10¹⁵ can be expected.

(5) Catalytically important amino acids are spaced along the entire polypeptide chain of a protein. During folding and the formation of the three-dimensional structure, catalytically important functional groups become close in space and thus form the three-dimensional structure of the catalytic centre.

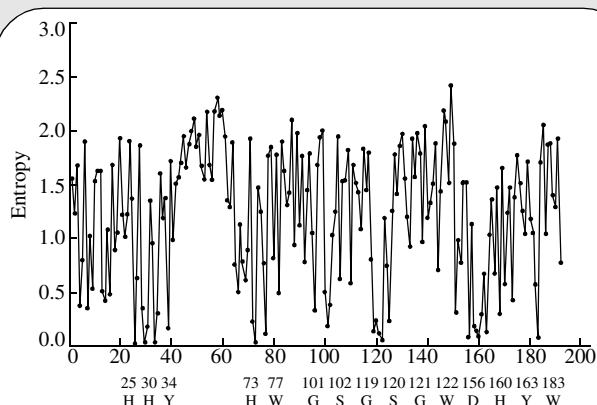


Figure 1 Shannon's entropy calculated for multiple alignment positions for proteins of the Fe²⁺-dependent superoxidedismutase family (371 compared proteins).

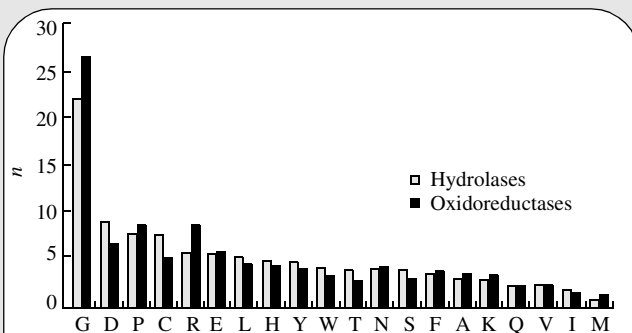


Figure 2 Conservatism rating of amino acids for hydrolases and oxidoreductases; n is the occurrence of amino acids as conservative (%).

Proper folding of the polypeptide chain is ensured by the presence of structure-forming amino acids (glycine, proline, and eventually cysteine) at proper positions. However, the order of catalytically important amino acids in a polypeptide chain can vary. For example, two catalytic proteases, chymotrypsin (a protein of animal origin) and subtilisin (a protein from micro-organisms) have the following sequences of amino acids that form the catalytic triads: His57, Asp102, Ser195 (chymotrypsin) and Asp32, His64, Ser221 (subtilisin). However, the active centres are structurally identical in the three-dimensional structures of catalytic centres formed from quite different polypeptide chains. Two different sequences of catalytic groups result in the formation of two different secondary structures. All serine proteases in which the catalytic groups are arranged by the α -chymotrypsin type are β -proteins. All proteases in which the catalytic groups are arranged by the subtilisin type are α / β -proteins according to the classification of secondary structures.

(6) The main structural paradox of enzyme catalysis is that the huge number of catalytic reactions involving a virtually infinite number of proteins differing in primary structures is based on quite a limited number of catalytic centres.

For example, hydrolases are the most numerous class of enzymes counting about one-third of all the currently known enzymes (about 1100 enzymes from 3700 in accordance with the enzyme classification). Each of these enzymes is subject to biological variety since these proteins are represented by a virtually infinite number of variants of amino acid sequences in biological systems. However, there are only five main catalytic centres that perform activation and a catalytic cycle.^{5,9} The diversity of reactions (about 1100 for hydrolases) is due to the action of the binding centre, which performs the complexation of a substrate and orientation of the reaction centre relative to the catalytic groups of the protein. Thus, catalytic centres in enzymes are unique structures that have appeared during evolution, probably, as a result of trying numerous intermediate structures, and have been fixed at a genetic level as certain positions of catalytically important amino acids and structure-forming amino acids, which gather the functional groups of catalytic amino acids in the required point of space and with a required steric conformance.

(7) The phenomenon of structural unity of construction of catalytic centres manifests itself when active centres of enzymes other than hydrolases are compared with those of hydrolases.

Analysis shows that active centres of enzymes other than hydrolases are built from the same nucleophilic–electrophilic units as the active centres of hydrolases. The reaction centres of molecules are activated by the same nucleophilic–electrophilic mechanisms and the activation frequently involves the same three-dimensional structures.

For example, it occurs very often that the charge transfer chain comprising the imidazole–carboxyl group pair can be identified in the active centres of oxidoreductases. The His194–Asp165 pair, which performs the nucleophilic activation of lactic acid, can be found in the active centre of lactate dehydrogenase, as well as in those of many NAD⁺-dependent dehydrogenases. Proton transfer along the hydrogen bond between the imidazole

group of histidine 194 and the hydroxy group of lactic acid induces a negative charge on the reaction centre (the α -hydrogen atom), which favors hydride transfer to the NAD⁺ molecule. The positively charged guanidine moiety Arg105 is the electrophilic mediator in this transfer.

In peroxidases, *i.e.*, enzymes that use hydrogen peroxide for the oxidation of various molecules and contain heme as a prosthetic group, an imidazole group (*e.g.*, His175 for cytochrome C-peroxidase) serves as a ligand for the iron ion. The positively charged iron ion interacts with the unshared electron pair of the imidazole group to form a strong coordination bond. On the other hand, the imidazole group is connected by a hydrogen bond (through the second nitrogen atom) with the carboxy group of aspartic acid Asp135. As in hydrolases, imidazole is a mediator of electron density transfer; in this case, it performs the polarizing partial charge transfer from the negatively charged carboxy group to the metal ion. Like in the triad of serine hydrolases (dehydrogenases), peroxidases contain a reactive dynamically mobile nucleophilic agent, which consists of a structurally organised pair ‘histidine imidazole group–carboxy group of aspartic acid’. As in the case of dehydrogenases, the catalytic cycle involves the guanidine moiety of arginine (Arg48), which favours electron transfer from the substrate to the peroxide through the iron ion in oxidised form.

(8) The phenomenon of structural unity of active centres in enzymes becomes even more obvious when one compares the structures of active centres of hydrolases with the corresponding structures of synthetases, which perform the formation of bonds and do not involve a water molecule as a reagent. In this case, the transition from hydrolytic to synthetic reactions is carried out by the replacement of water activation to the activation of, *e.g.*, the hydroxy group of a carbohydrate with total retention of the structure of the activating centre.

The catalytic centre of exonucleases, which hydrolyse nucleic acids, is a complex of magnesium ions formed with the participation of carboxy groups of aspartic and glutamic acids (the pyrophosphatase type). The active centre of the enzyme that synthesizes DNA, *i.e.*, DNA-polymerase, is highly homologous to that of nuclease, *i.e.*, the enzyme that hydrolyses DNA. The nucleophile reaction centre in DNA-polymerase is activated by the same functional groups as in pyrophosphatase or exonuclease. However, instead of the activation of water, a carbohydrate hydroxy group is activated in DNA-polymerase. A nucleophilic attack on the reaction centre of the phosphate group ensures the formation of a phosphodiester bond and continuation of the DNA chain. Thus, here we can see an example of transition from enzymes that hydrolyse bonds to enzymes that create such bonds. Structural replacement of water in the active centre by another possible nucleophile is sufficient for creating an active centre that can perform a synthetic reaction.

The identification of catalytic and structure-forming amino acids from the primary sequence of amino acids in the polypeptide chain as described above and the phenomenon of structural unity of enzymes belonging to different classes have many consequences.

Obviously, proteins with catalytic activity can be constructed from fewer amino acids than the twenty that occur in nature. The acids in the majority of positions in a polypeptide chain can be replaced by other amino acids without changing considerably the catalytic properties of the protein. It is important that certain positions contain a number of critical amino acids, either amino acids with acid–base properties that form the catalytic centre or structure-forming amino acids that assemble the catalytic groups in a certain point in space.

Traditional methods for modifying enzyme properties include the site-directed mutagenesis or the directed evolution method.⁵ Results of the analysis performed show that amino acids can be varied freely only at positions that are not conservative. Otherwise, there is a high probability for a modified enzyme to lose its enzyme activity.

Obviously, the type of catalytic reactions involving an enzyme is largely determined by the structure of the binding centre,

which ensures the position for an attack by catalytic groups. This creates prospects for developing protein catalysts for reactions unknown in the biological world; thus, the applicability of enzymes for solving chemical problems may be expanded considerably. The current level of development in chemical enzymology makes it possible to set the problem of creating enzymes for a required chemical reaction that is not a natural reaction.

As an example, we describe below such a reaction. We have shown that enzymes can be efficient catalysts for the oxidation of metals by atmospheric oxygen or hydrogen peroxide (experimental data by I. K. Sakodinskaya) and, in this case, the enzymatic reaction can be transported through an electronic conductor.

We have shown previously that enzymes can act as the catalysts of electrochemical reactions when they are adsorbed on electronic conductors.^{17–20} Adsorption of blue copper-containing oxidase (laccase) on an inert electrode shifts the potential of the latter to the oxygen ionization potential (~1.2 V vs. a hydrogen electrode in the same solution), while the adsorption of hydrogenase shifts the potential to the hydrogen ionization potential. Immobilised peroxidase shows a similar acceleration of the electrochemical reaction of hydrogen peroxide reduction. In all these cases, direct electron transfer occurs between the electrically conductive matrix and the active centre of the enzyme. Theoretical calculations and experimental data show that the ‘electrical connection’ between the electronic conductor and the active centre of the enzyme is formed due to the relatively fast transfer of an electron by the tunneling mechanism.²¹ The phenomenon of acceleration of electrode processes by enzymes involving the mechanism of electronic contacts between a conductor and an enzyme active centre was called bioelectrocatalysis.²² Bioelectrocatalysis finds various applications, primarily, in the construction of biosensors^{23–25} and fuel cells.^{26–29}

Bioelectrocatalysis was studied using corrosion-resistant electronic conductors, such as carbon and gold. It could be expected that, if corroding metals were used, enzymes would accelerate the oxidation of such metals. We have found that laccase is a catalyst of oxidation of metals when oxygen is used as an oxidant, whereas peroxidase behaves similarly when hydrogen peroxide is used. Enzymes efficiently accelerate the oxidation of iron, steel, stainless steel and copper.

It is of fundamental interest to determine the mechanism of the oxidation processes. In particular, the question is whether metal ionization (transition of a metal from neutral to ionized form) occurs directly in the zone of action of the enzyme active centre or the transfer of electrons to the oxidant *via* the active centre and abstraction of a metal ion from the substrate are spatially separated. To answer this question, we designed a cell consisting of two spatially separated vessels connected by a salt bridge and a metallic conductor (a copper wire). An enzyme (laccase) was added to one of the vessels and an equivalent volume of the corresponding buffer solution was added to the other vessel. The formation and accumulation of metal ions was observed in both vessels; sometimes, this occurred faster in the enzyme-free vessel than in the vessel containing the enzyme. This suggests that the enzyme adsorbed on the metal surface works as a catalyst of electron transfer from the metal substrate to oxygen, whereas the electron vacancy formed manifests itself as the transition of a metal ion to the solution from a metal surface in a place remote from the location of the enzyme molecule. This experiment demonstrates the amazing prospects of transferring an enzymatic reaction through electric wires.

References

- 1 I. V. Berezin and K. Martinek, *Osnovy fizicheskoi khimii fermentativnogo kataliza* (Fundamentals of Physical Chemistry of Enzymatic Catalysis), Vysshaya Shkola, Moscow, 1977 (in Russian).
- 2 V. K. Antonov, *Khimiya proteoliza* (Chemistry of Proteolysis), Nauka, Moscow, 1991 (in Russian).
- 3 M. L. Bender, *Mechanisms of Homogeneous Catalysis from Proton to Proteins*, Wiley-Interscience, New York, 1971.
- 4 W. P. Jencks, *Catalysis in Chemistry and Enzymology*, McGraw Hill, New York, London, Mexico, 1969.
- 5 S. D. Varfolomeev, *Khimicheskaya enzimologiya* (Chemical Enzymology), Akademiya, Moscow, 2004 (in Russian).
- 6 S. D. Varfolomeev and S. V. Zaitsev, *Kineticheskie metody v biokhimicheskikh issledovaniyakh* (Kinetic Methods in Biochemical Studies), Izdatel'stvo Moskovskogo Universiteta, Moscow, 1982 (in Russian).
- 7 S. D. Varfolomeev and K. G. Gurevich, *Biokinetika* (Biokinetics), Fair-Press, Moscow, 1999 (in Russian).
- 8 S. D. Varfolomeev, K. G. Gurevich, V. V. Poroikov, B. N. Sobolev and A. E. Fomenko, *Dokl. Ross. Akad. Nauk*, 2001, **379**, 548 [*Dokl. Biochem. (Engl. Transl.)*, 2001, **379**, 252].
- 9 S. D. Varfolomeev and K. G. Gurevich, *Izv. Akad. Nauk, Ser. Khim.*, 2001, 1629 (*Russ. Chem. Bull., Int. Ed.*, 2001, **50**, 1709).
- 10 S. D. Varfolomeev, I. V. Uporov and E. V. Fedorov, *Biokhimiya*, 2002, **67**, 1099 [*Biochemistry (Moscow)*, 2002, **67**, 1328].
- 11 <http://www.expasy.org>
- 12 <http://seop.mrc-lmb.cam.ac.uk/scop/index.html>
- 13 <http://www.sander.ebi.ac.uk/hssp/>
- 14 <http://www.rcsb.org>
- 15 A. Finkelshtein, *Fizika belka* (Physics of Proteins), Nauka, Moscow, 2002 (in Russian).
- 16 S. D. Varfolomeev, I. A. Gariev and I. V. Uporov, *Usp. Khim.*, 2005, in press.
- 17 I. V. Berezin, V. A. Bogdanovskaya, S. D. Varfolomeev, M. R. Tarasevich and A. I. Yaropolov, *Dokl. Akad. Nauk SSSR*, 1978, **240**, 615 [*Dokl. Phys. Chem. (Engl. Transl.)*, 1978, **240**, 455].
- 18 S. D. Varfolomeev and S. O. Bachurin, *J. Mol. Catal.*, 1984, **27**, 315.
- 19 S. D. Varfolomeev, A. I. Yaropolov and A. A. Karyakin, *Biotechnology*, 1993, **27**, 331.
- 20 A. I. Yaropolov, O. V. Skorobogat'ko, S. S. Vartanov and S. D. Varfolomeev, *Appl. Biochem. Biotechnol.*, 1994, **49**, 257.
- 21 A. I. Yaropolov, T. K. Sukhomlin, A. A. Karyakin, S. D. Varfolomeev and I. V. Berezin, *Dokl. Akad. Nauk SSSR*, 1981, **260**, 1192 [*Dokl. Phys. Chem. (Engl. Transl.)*, 1981, **260**, 961].
- 22 S. D. Varfolomeev, M. R. Tarasevich, A. I. Yaropolov, I. V. Berezin and V. A. Bogdanovskaya, *USSR State Registry of Discoveries*, Discovery no. 311, December 25, 1987.
- 23 S. D. Varfolomeev, I. N. Kurochkin and A. I. Yaropolov, *Biosens. Bioelectronics*, 1996, **11**, 863.
- 24 A. A. Karyakin, E. E. Karyakina, W. Schuhmann, H.-L. Smidt and S. D. Varfolomeev, *Electroanalysis*, 1994, **6**, 821.
- 25 L. A. Lukachova, A. A. Karyakin, Y. N. Ivanova, E. E. Karyakina and S. D. Varfolomeev, *Analyst*, 1998, **123**, 1981.
- 26 S. D. Varfolomeev and A. I. Yaropolov, in *From Neural Networks and Biomolecular Engineering to Bioelectronics*, ed. C. N. Nicolini, Plenum Press, New York, 1995, p. 121.
- 27 S. V. Morozov, E. E. Karyakina, N. A. Zorin, S. D. Varfolomeev and S. Cosner, *Bioelectrochem.*, 2002, **55**, 169.
- 28 A. A. Karyakin, S. V. Morozov, E. E. Karyakina, S. D. Varfolomeev, N. A. Zorin and S. Cosner, *Electrochem. Commun.*, 2002, **4**, 417.
- 29 A. A. Karyakin, S. V. Morozov, E. E. Karyakina, M. Yu. Vagin and S. D. Varfolomeev, *RF Patent no. 2003 11 2989/13* (014144), 06.05.2003.

Received: 30th August 2004; Com. 04/2345