Metal Ions in Biology and Medicine: vol. 10. Eds Ph. Collery, I. Maymard, T. Theophanides, L. Khassanova, T. Collery. John Libbey Eurotext, Paris © 2008 pp. 522-527.

Probing cation-binding sites in metalloproteins using ^{57}Co emission Mössbauer spectroscopy. New developments, potentials and prospects

Alexander A. Kamnev

Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, 410049 Saratov, Russia; e-mail: aakamnev@ibppm.sgu.ru

ABSTRACT

The highly sensitive and informative emission variant of Moessbauer (nuclear (-resonance) spectroscopy (EMS; using the ^{57}Co radionuclide) has recently been shown for the first time to be applicable to probing cation-binding sites of the enzyme active centres. As the first example of a tested enzyme, in our studies we used glutamine synthetase (GS, a key enzyme of nitrogen metabolism, which is ubiquitous in all organisms from bacteria to humans), isolated from diazotrophic rhizobacterium *Azospirillum brasilense*. The reasons for choosing this particular enzyme for testing the validity of the data obtained by EMS, as well as some important methodological prerequisites for using the technique are discussed. Further developments, potentials and prospects of ^{57}Co EMS are considered with the aim to broaden the scope of possible applications of this uncommon technique in biochemistry and related biological fields.

Key words: emission moessbauer spectroscopy, ^{57}Co radionuclide, cobalt biocomplexes, cation-binding sites, catalytic active centers, metalloenzymes, metalloproteins

INTRODUCTION

Moessbauer (nuclear (-resonance) spectroscopy in the traditional absorption variant, with the stable ^{57}Fe isotope being most widely used, has had a rich history of applications in a broad range of fields, including life sciences (for recent reviews see, e.g. [1-4]). As for the ^{57}Fe absorption variant of Moessbauer spectroscopy, the main limitations are: (*i*) a relatively low abundance of ^{57}Fe in the natural iron (2.19%), so that samples to be studied containing only traces of Fe have to be enriched with ^{57}Fe; (*ii*) a relatively low sensitivity (i.e., with minimal sample requirements of > 1 mM ^{57}Fe and a sample volume about 0.3-0.4 ml [3]). As the Moessbauer effect (i.e., recoilless absorption or emission of γ-quanta) can be observed in a solid matrix only, solutions or liquid samples are commonly studied rapidly frozen [1,3,4].

The incomparably more sensitive and very informative emission variant of Moessbauer spectroscopy (EMS; with the ^{57}Co radionuclide as the most widely used isotope, having a half-life of ca. 9 months) requires several orders of magnitude lower amounts of ^{57}Co in a sample to obtain a good emission spectrum (some features and applications of this peculiar technique have been outlined recently in a specialised issue of the Moessbauer Effect Reference and Data Journal [5]). Owing largely to the evident specific methodological difficulties of this nuclear chemistry technique, the number of yearly EMS publications have tended to decline from a maximum of about a hundred papers per year (reached in the 70'ies of the 20th century) down to ca. 20 papers per year by the 21st century (see [5], p. 90). In life sciences, applications of ^{57}Co EMS have so far been featured by a few reports only (see, e.g. [6-8] and references therein).

The ^{57}Co EMS technique has recently been shown for the first time to be applicable to probing cation-binding sites of the enzyme active centres [9]. In this communication, we review the progress

and discuss this novel approach considering further developments, potentials and future prospects of the ^{57}Co EMS methodology, with the aim to broaden the scope of possible applications of this uncommon technique in biochemistry and related biological fields.

NOVEL APPLICATIONS IN ENZYMOLOGY

The choice of a test object

As the first example of a metal-dependent enzyme tested with ^{57}Co EMS, in our studies [9,10] we used a bacterial glutamine synthetase (GS, EC 6.3.1.2; a key enzyme of nitrogen metabolism, which is ubiquitous in all organisms from bacteria to humans [11]). For instance, in humans, GS is expressed in tissues, being involved in ammonia detoxification and interorgan nitrogen flux; its malfunctioning or inherited deficiency was found to lead to multiple disorders of organs and brain malformation [11,12].

There were logical reasons for choosing this particular enzyme, isolated from the soil diazotrophic bacterium *Azospirillum brasilense* (reviewed recently in [13]), for testing the validity of data obtained by the EMS technique. A few main reasons are considered below.

First, cobalt(II) is among its activating divalent cations, along with Mg^{2+} and Mn^{2+} [13,14]. It has been shown that the enzyme can be obtained in a cation-free state (by removing the native cation(s) bound in the active centres using EDTA treatment with subsequent dialysis), in which it loses its biocatalytic activity in the absence of divalent cations in the medium [14]. Adding Co^{2+} to the inactive metal-free GS has been found to reinstate its activity [15], thereby proving that the added Co^{2+} cations bind in the active centres (the latter is prerequisite for the GS activity to be expressed [11,13,14]). Thus, doping the cation-free GS with a precalculated amount of $^{57}Co^{2+}$ under physiologically similar conditions in principle allows ^{57}Co EMS to be applied.

Second, this enzyme has *two* cation-binding sites (n1 and n2) at each of its 12 active centres (i.e., total 24 sites) per molecule [11] *(Figure 1)*. From the structural point of view, a molecule of bacterial GS is a dodecamer formed from two hexameric rings of subunits (monomers; see *Figure 1a*) disposed face-to-face; each of the 12 active centres (having the shape of a'bifunnel'; see *Figure 1b*) is located between every two neighbouring subunits within a ring. Site n1 has ca. 50-fold greater affinity for cations than site n2, which is related to their coordination modes and charge distribution [11]. In site n1, the cation is coordinated by three glutamic acid (Glu) residues (E131, E212, E220), i.e. by their three side-chain carboxylates (with possible additional binding of H_2O [16]), while in site n2 by two Glu (E129, E357) and one histidine, H269 (i.e., one N-donor atom of the His heterocycle and two Glu carboxyls), and this structure is strictly conserved among different GSs [11]. The cations in the two sites are 0.6 nm apart, have no common ligands (i.e., not bridged) and therefore, from the spectroscopic point of view, may be regarded as relatively independent from each other. Thus, sites n1 and n2, when both occupied by $^{57}Co^{2+}$ cations, could be expected to exhibit different Moessbauer parameters in an emission spectrum (i.e., giving distinguishable spectral components) owing to their essentially different coordination microenvironments. Moreover, since the affinity for cations is much higher in site n1, in case when the molar $^{57}Co^{2+}$-to-GS ratio (x) is made higher than that required to saturate half of all the sites (i.e., primarily sites n1) but under the'saturation limit' for both n1 + n2 (i.e., under the condition $12 < x < 24$), the resulting ratio of the areas for the subspectra (spectral components) corresponding to n1 and n2 could be expected to allow $^{57}Co^{2+}$ distribution between the sites to be assessed (see *Figure 1c*).

Despite the fact that some important structural information for GSs from a few pathogenic enterobacteria has already been reported [11,17] (showing, in particular, the same shape of the active site cavity and the cation-binding amino acid residues therein) and there are also some data on *A. brasilense* GS (see [13,14] and references cited therein), it is obvious that further structural investigations of this enzyme are vitally necessary. On one hand, even bacterial (type I) GSs significantly differ from each other by their regulation and a number of other properties, and the *A. brasilense* GS shows yet more differences in its regulatory mechanisms [13], still leaving much

to be understood. On the other hand, eukaryotic (type II) GSs, being significantly different from type I forms of GS (except for the invariable set of amino acid residues within the catalytic sites), are significantly less studied, and further information would shed light on the mechanisms underlying a number of diseases related to GS functioning [11,12].

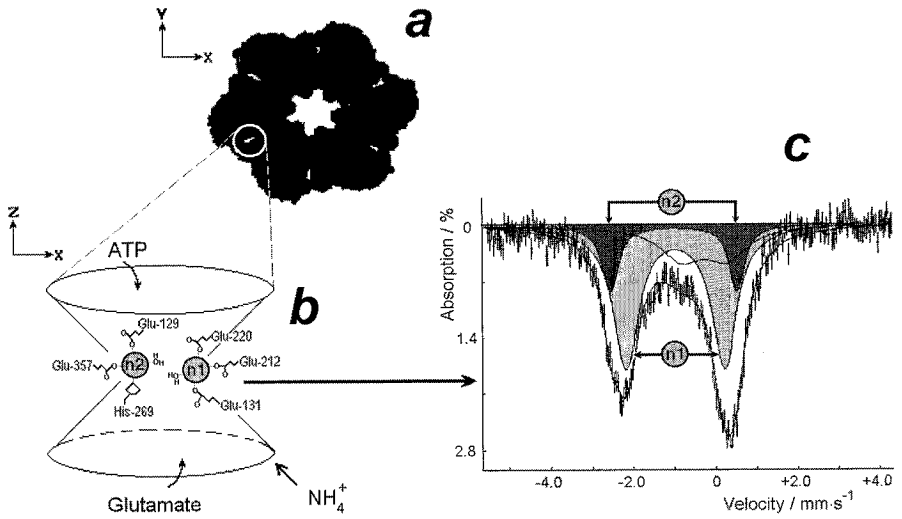

Figure 1. Schematic presentation of ***(a)*** a top view of one of the two hexameric rings of a bacterial glutamine synthetase (GS) molecule (the position of an active centre in an intersubunit space is shown by white circle); ***(b)*** a side-view of the active centre ('bifunnel', ca. 1.5 nm wide and 4.5 nm high, with metal ions in two cation-binding sites, n1 and n2), which ATP and glutamate enter at opposite ends as shown [11]. ***(c)*** An emission Moessbauer spectrum of cation-free GS from *Azospirillum brasilense* (in an average adenylylation state of 18%) doped with $^{57}Co^{2+}$ (measured in rapidly frozen aqueous solution; $T = 80$ K) [9,10]. The subspectra featuring sites n1 (light-shaded doublet) and n2 (dark-shaded doublet) are indicated by accordingly marked arrows (the third broader doublet of lower intensity is due to after-effects of the $^{57}Co \rightarrow ^{57}Fe$ nuclear transformation [9]). Relative resonant absorption of γ-quanta (in%) by a standard absorber ($K_4Fe(CN)_6 \cdot 3H_2O$) is plotted against relative velocity of the absorber *versus* the ^{57}Co-containing sample (γ-radiation source) [8], which corresponds to the energy scale according to the Doppler effect (±1 mm(s^{-1} corresponds to ±48.1 neV [3]), calibrated using α-Fe at room temperature.

Prerequisites for using the ^{57}Co EMS technique

While the enzyme active centres doped with $^{57}Co^{2+}$ can be probed using EMS as discussed above, nevertheless, for a correct analysis of the EMS data to be obtained, several conditions have to be observed [15]. First, it is important to make sure that the $^{57}Co^{2+}$ cations, substituting for the native metal(II) ions, are indeed bound within the active centres. If this is not so, the existence of numerous binding sites and, consequently, many forms of $^{57}Co^{II}$ bound to functional groups of the protein macromolecule would render the EMS data hardly interpretable. Second, the replacement of the native activating cations (e.g., by natural Co^{2+} under identical conditions) ideally should not result in an irreversible deactivation of the enzyme. In the latter case, the correspondence between the $^{57}Co^{II}$ form in the enzyme sample under study and the cobalt(II) form in the physiologically active enzyme would be doubtful. Finally, the quantity of the substituted $^{57}Co^{2+}$ should conform with the overall number of the cation-binding sites in the enzyme sample. Any excessive $^{57}Co^{2+}$, binding to different functional groups of the protein beyond the active centres, would evidently lead to an unpredictable complication of the spectra.

As the affinity for cations in the enzyme active centres is usually much higher than elsewhere on the protein globule, fulfilment of the above-mentioned conditions may well be feasible. Mo-

reover, when the active centre contains more than one cation-binding site with different affinities for the cation (but high, as compared to any other possible binding sites beyond active centres) and different coordination environments (as in the case with glutamine synthetase [11,13]), it may be expected that ^{57}Co EMS would allow one to obtain information not only on the chemical forms and coordination symmetry of the cobalt in each of the sites but also on its distribution between them. The above-discussed properties of *A. brasilense* GS were found to be suitable for using EMS in studying $^{57}Co^{2+}$-doped enzyme preparations [7-10,15].

FURTHER DEVELOPMENTS, POTENTIALS AND PROSPECTS

Two-metal-ion catalysis: competitive metal binding at the enzyme active centres

Besides GS (both types I and II), a number of other metal-dependent enzymes including, e.g. alkaline phosphatases, endonucleases, DNA/RNA polymerases, etc., require two metal cations bound in the active centre for their catalytic activity [13,18]. It has, however, to be noted that while, for instance, in polymerases and nucleases the two cations are coordinated *jointly* by both a conserved Asp residue and the scissile phosphate [18], in GS the two cations are bound by a few *separate* amino acid residues (see above and *Figure 1b*). Moreover, the GS substrates bind to different cations (glutamate to n1 and ATP to n2; NH_4^+ has its separate binding site close to n1) [11]. This is in line with a relatively large distance between the two cations in GS (0.6 nm [11]), as compared to those in, e.g. RNases (0.4 to 0.3 nm [18]).

While for another bacterial GS – the product of the *glnA1* gene from *Mycobacterium tuberculosis* (expressed in *E. coli*) – the possibility of the simultaneous binding of two *different* metal cations (Mg^{2+} and Mn^{2+}) at sites n1 and n2 has been considered [17], for *A. brasilense* GS this has so far been unclear. As for the terminology (keeping in mind also the possible different modes of coordination of the cations both to protein residues and to substrates), two-metal-ion binding may be related to *binuclear* catalysis. Thus, binding of *the same* metal cation in both sites of an active centre may be related to *homobinuclear* catalysis, whereas *different* metal cations binding at the two sites of an active centre may be related to *heterobinuclear* catalysis.

Preliminary comparative experimental studies were performed on partly adenylylated GS from *A. brasilense* (in an intermediate adenylylation state of 44%) doped with $^{57}Co^{2+}$ alone or together with natural Mn^{2+} (the latter gives no contribution to emission spectra) [19]. Adding equimolar quantities of $^{57}Co^{2+}$ + Mn^{2+} to the metal-free enzyme, as compared to $^{57}Co^{2+}$ alone, was found to result in a partial redistribution of $^{57}Co^{II}$ between the sites, showing competitive binding of the two cations. This finding, indirectly pointing to the possibility of heterobinuclear catalysis in bacterial GS, is in agreement with similar efficiency of the two cations in supporting the activity of partly adenylylated GS from *A. brasilense* [14]. Further ^{57}Co EMS studies are therefore warranted in order to compare not only the enzyme's specificity for different cations [13,14], but also their'preference' for each of the two different sites (n1 and n2), possible competitive binding and redistribution within the GS active centres.

Coordination symmetry at cation-binding sites

The Moessbauer parameters obtained from both ^{57}Fe absorption and ^{57}Co emission spectra are sensitive not only to the chemical nature of metal-ligand bonds but also to their symmetry [1-4]. Moreover, the data obtained in ^{57}Co EMS experiments may be regarded as a'snapshot' of daughter ^{57}Fe atoms appearing in the microenvironment of the parent ^{57}Co species. Thus, a comparison between ^{57}Co EMS data for a cobalt complex and absorption ^{57}Fe Moessbauer data for an iron complex with the same ligands sometimes can give valuable additional information.

The EMS data obtained for ^{57}Co-doped GS in an adenylylation state of 18% (see *Figure 1c*) showed the following parameters for sites n1 and n2, respectively: isomer shift $\delta = 1.05 \pm 0.02$ and 1.08 ± 0.02 mm·s^{-1} (*versus* α-Fe; converted to the absorption convention; 80 K), quadrupole

splitting $\Delta = 2.39 \pm 0.06$ and 3.08 ± 0.08 mm·s^{-1} [9] (note that statistically indistinguishable data were obtained for the same sample in the dried state at $T = 80$ K, showing the invariability of the cobalt(II) coordination at both sites of the enzyme in both states, which corresponds to the known fact that GS retains its properties when stored in the dried state [10]). While these Δ values, together with the relative areas of the subspectra (see *Figure 1c*), allowed sites n1 (with a higher affinity) and n2 (with a lower affinity, as well as with lower coordination symmetry owing to different coordinated amino acid residues) to be distinguished, the δ values for both sites correspond to tetrahedral (T_d) coordination of the parent $^{57}Co^{II}$.

For comparison, the δ values reported for several high-spin carboxylate-rich Fe^{II} complexes (some with N-donor atoms; measured at $T = 4.2$ K) are within the range $(1.04 \div 1.08) \pm 0.02$ mm·s^{-1} for the T_d geometry of the sites and $(1.26 \div 1.35) \pm 0.02$ mm(s^{-1} for the octahedral (O_h) geometry [20]. Complexes of $^{57}Co^{II}$ with anthranilic (*o*-aminobenzoic) acid and L-tryptophan, studied using ^{57}Co EMS in frozen aqueous solutions, showed, respectively, $\delta = 1.10 \pm 0.02$ and $\delta = 0.88 \pm 0.06$ mm·s^{-1}, $\Delta = 2.71 \pm 0.05$ and 2.8 ± 0.1 mm·s^{-1} [21], which is also within the range for high-spin T_d coordination. Note that solid Fe^{II} anthranilate Fe(*anthr*)$_2$ with an O_h coordination (with two *bidentate* carboxylates and two amino groups as ligands) gave $\delta = 1.25 \pm 0.01$ mm·s^{-1} (at $T = 80$ K) [21]. Thus, the T_d geometry at the cation-binding sites of the GS sample [10,21] evidently corresponds to monodentate binding of all the Glu carboxylates involved. It may be expected that any intermediate coordination state of the activating cations, e.g. occurring during substrate turnover, could be distinguished using a rapid freeze-quench variant [3] of ^{57}Co EMS.

Possibilities of ^{57}Co substitution for other cations in metalloproteins

Cobalt(II) acts as an activating cation or a cofactor in a variety of enzymes, which could thus be directly probed using ^{57}Co doping and EMS. Moreover, Co^{2+} has also been shown to be applicable as an *isostructural substitute*, e.g. for Zn^{2+} in many zinc-containing proteins (see [22] and references therein). Besides the applicability of Co^{2+} as an optical (UV-Vis) probe that gives clearly different spectra for, e.g. T_d and O_h coordination, $^{57}Co^{2+}$ substitution for other cations can give a highly sensitive "snapshot" of the coordination microenvironment and its fine structural changes when used in EMS. This possibility could expand the areas of ^{57}Co EMS applications beyond only cobalt-dependent metalloproteins and enzymes, greatly enhancing the importance of the technique for biochemistry, molecular biology and related fields. Note also that ^{57}Co EMS can be useful in studies on metal sorption by microbial cells, as well as on bioleaching and transformations of heavy metals and radionuclides mediated by soil microorganisms [7,8].

Acknowledgements: The author is grateful to Prof. Yu.D. Perfiliev, Dr. L.A. Kulikov (Moscow, Russia), Prof. A. Vertes and Prof. E. Kuzmann (Budapest, Hungary) for many stimulating discussions and long-term fruitful collaboration, as well as to D.Sc. L.P. Antonyuk and Dr. V.E. Smirnova (Saratov, Russia) for sharing their expertise in biochemistry and enzymology. This work, comprising the material submitted to the 10th ISMIBM as part of an invited lecture, was supported within the recent years under the NATO "Security Through Science" Programme (Collaborative Linkage Grants LST.CLG.977664, LST.NR.CLG.981092 and ESP.NR.NRCLG 982857; Expert Visit Grants LST.EV.980141 and CBP.NR.NREV 981748) and under the Agreement on Scientific Cooperation between the Russian and Hungarian Academies of Sciences for 2005-2007.

REFERENCES

1. Kuzmann E., Homonnay Z., Nagy S., Nomura K. Moessbauer spectroscopy. In A. Vertes, S. Nagy, Z. Klencsar (Eds.), *Handbook of Nuclear Chemistry*, Vol. 3. Kluwer, Dordrecht, 2003, pp. 109-87.
2. Oshtrakh M.I. Study of the relationship of small variations of the molecular structure and the iron state

in iron containing proteins by Moessbauer spectroscopy: biomedical approach. *Spectrochim Acta Part A: Mol Biomol Spectrosc* 2004; 60: 217-34.
3. Krebs C., Price J.C., Baldwin J., Saleh L., Green M.T., Bollinger J.M., Jr. Rapid freeze-quench ^{57}Fe Moessbauer spectroscopy: Monitoring changes of an iron-containing active site during a biochemical reaction. *Inorg Chem* 2005; 44: 742-57.
4. Semenov V.G., Moskvin L.N., Efimov A.A. Analytical potential of Moessbauer spectroscopy. *Russ Chem Rev* (Engl Transl) 2006; **75**: 317-27.
5. Editor's comments, Emission Moessbauer publications, Moessbauer Spectroscopy Newsletter. *Moessbauer Effect Ref & Data J* 2007; 30 (5): 89, 90, 107-22.
6. Nath A. Electronic relaxation following an Auger event: Whither goes colossal excitation energy? Fall-out for emission Moessbauer spectroscopy. *Moessbauer Effect Ref & Data J* 2007; 30 (5): 107-109.
7. Perfiliev Yu.D., Kamnev A.A. Emission Moessbauer spectroscopy. *Moessbauer Effect Ref & Data J* 2007; 30 (5): 121-22.
8. Kamnev A.A. Application of emission (^{57}Co) Moessbauer spectroscopy in bioscience. *J Mol Struct* 2005; 744-747: 161-67.
9. Kamnev A.A., Antonyuk L.P., Smirnova V.E., Serebrennikova O.B., Kulikov L.A., Perfiliev Yu.D. Trace cobalt speciation in bacteria and at enzymic active sites using emission Moessbauer spectroscopy. *Anal Bioanal Chem* 2002; 372: 431-35.
10. Kamnev A.A., Antonyuk L.P., Smirnova V.E., Kulikov L.A., Perfiliev Yu.D., Kudelina I.A., Kuzmann E., Vertes A. Structural characterization of glutamine synthetase from *Azospirillum brasilense*. *Biopolymers* 2004; 74: 64-8.
11. Eisenberg D., Gill H.S., Pfluegl G.M.U., Rotstein S.H. Structure-function relationships of glutamine synthetases. *Biochim Biophys Acta* 2000; 1477: 122-45.
12. Haberle J., Gorg B., Toutain A., Rutsch F., Benoist J.-F., Gelot A., Suc A.-L., Koch H.G., Schliess F., Haussinger D. Inborn error of amino acid synthesis: Human glutamine synthetase deficiency. *J Inherit Metab Disease* 2006; 29: 352-58.
13. Antonyuk L.P. Glutamine synthetase of the rhizobacterium *Azospirillum brasilense*: specific features of catalysis and regulation. *Appl Biochem Microbiol* (Moscow) 2007; 43: 244-49.
14. Antonyuk L.P., Smirnova V.E., Kamnev A.A., Serebrennikova O.B., Vanoni M.A., Zanetti G., Kudelina I.A., Sokolov O.I., Ignatov V.V. Influence of divalent cations on the catalytic properties and secondary structure of unadenylylated glutamine synthetase from *Azospirillum brasilense*. *BioMetals* 2001; 14: 13-22.
15. Kamnev A.A., Antonyuk L.P., Kulikov L.A., Perfiliev Yu.D., Kuzmann E., Vertes A. Probing enzyme active centers doped with $^{57}Co^{2+}$ ions using emission Moessbauer spectroscopy. *Bull Russ Acad Sci Physics* (Engl Transl) 2005; 69: 1561-65.
16. Eads C.D., LoBrutto R., Kumar A., Villafranca J.J. Identification of nonprotein ligands to the metal ions bound to glutamine synthetase. *Biochemistry* (US) 1988; 27: 165-70.
17. Gill H.S., Pfluegl G.M.U., Eisenberg D. Multicopy crystallographic refinement of a relaxed glutamine synthetase from *Mycobacterium tuberculosis* highlights flexible loops in the enzymatic mechanism and its regulation. *Biochemistry* (US) 2002; 41: 9863-72.
18. Yang W., Lee J.Y., Nowotny M. Making and breaking nucleic acids: Two-Mg^{2+}-ion catalysis and substrate specificity. *Mol Cell* 2006; 22: 5-13.
19. Kamnev A.A., Antonyuk L.P., Smirnova V.E., Kulikov L.A., Perfiliev Yu.D., Kuzmann E., Vertes A. Bacterial glutamine synthetase: Two-cation-bearing active centres of the enzyme probed by ^{57}Co emission Moessbauer spectroscopy. *FEBS J* 2005; 272 (Suppl. 1): 10.
20. Yoon S., Lippard S.J. Water affects the stereochemistry and dioxygen reactivity of carboxylate-rich diiron(II) models for the diiron centers in dioxygen-dependent non-heme enzymes. *J Amer Chem Soc* 2005; 127: 8386-97.
21. Kamnev A.A., Kulikov L.A., Perfiliev Yu.D., Antonyuk L.P., Kuzmann E., Vertes A. Application of ^{57}Co emission Moessbauer spectroscopy to studying biocomplexes in frozen solutions. *Hyperfine Interact* 2005; 165: 303-8.
22. Adamczyk M., Poznanski J., Kopera E., Pal W. A zinc-finger like metal binding site in the nucleosome. *FEBS Lett* 2007; 581: 1409-16.