



Application of emission (^{57}Co) Mössbauer spectroscopy in bioscience

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Abstract

Cobalt is an essential trace element with a broad range of physiological and biochemical functions. However, biochemical speciation of cobalt and structural investigations of cobalt-containing complexes with biomacromolecules are challenging, as the participation of cobalt in physiological processes is limited by its very low concentrations.

Emission Mössbauer spectroscopy (EMS), with the radioactive ^{57}Co isotope as the most widely used nuclide, is several orders of magnitude more sensitive than its ^{57}Fe absorption variant which has had a rich history of applications in bioscience. Nevertheless, owing to specific difficulties related to the necessity of using radioactive ^{57}Co in samples under study, applications of EMS in biological fields have so far been sparse. In this communication, the EMS applicability to studying biological objects as well as some specific aspects of the EMS methodology are considered in order to draw attention to the unique structural information which can be obtained non-destructively in situ. Chemical consequences (after-effects) of the nuclear transition ($^{57}\text{Co} \rightarrow ^{57}\text{Fe}$), which provide additional information on the electron acceptor properties of the proximate chemical microenvironment of the metal ions, are also considered. The data presented demonstrate that EMS is a sensitive tool for monitoring the chemical state and coordination of cobalt species in biological matter and in biomacromolecular complexes (metalloenzymes), providing valuable structural information at the atomic level.

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1. Introduction

Cobalt, among a variety of biologically essential trace elements and, in particular, transition metals, has a broad range of physiological and biochemical functions [1–7]. However, biochemical speciation of cobalt and structural investigations of cobalt-containing complexes with biomacromolecules are challenging, as the participation of cobalt in physiological processes is limited by its very low concentrations.

The emission variant of Mössbauer (nuclear gamma-resonance) spectroscopy (EMS), with the radioactive ^{57}Co isotope as the most widely used nuclide [8], is several orders of magnitude more sensitive than its ^{57}Fe absorption variant which has had a rich history of applications in bioscience (see, e.g. [9–13] and references therein). Nevertheless,

owing to specific difficulties related to the necessity of using radioactive ^{57}Co in samples under study, applications of EMS in biological fields have so far been sparse. In this communication, the EMS applicability to studying biological objects as well as some specific aspects of the EMS methodology are considered in order to draw attention to the unique structural information which can be obtained non-destructively in situ.

2. Methodology

The half-life period of the ^{57}Co radionuclide is 270 d. Its nuclear decay proceeding via electron capture gives the stable ^{57}Fe isotope and is accompanied by specific physical and chemical after-effects [8,14–16]. The resulting recoil energy (ca. 4.6 eV) for the daughter ^{57}Fe nucleus is sufficiently low, so that the nucleogenic iron atoms do not shift from their positions, and in many cases their chemical

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state and environment reflect that of the parent ^{57}Co species. However, as a result of the concatenated processes of consecutively filling in the vacancies in inner electronic shells of the ^{57}Fe atom after the electron capture by the ^{57}Co nucleus (the so-called Auger cascade developing within 10^{-15} to 10^{-14} s), Auger electrons are ejected out of the ^{57}Fe atom, giving a series of its different, mainly short-lived charge states. By the moment when a 14.4 keV γ -quantum is emitted by the nucleus (ca. 10^{-7} s after the electron capture), most of the Auger electrons return, giving common charge states of the daughter ^{57}Fe ions. Part of these states correspond to those of the parent ^{57}Co ; however, the interaction of some Auger electrons with the chemical environment of the atom can result in the formation of (an)other charge state(s) of the latter. For example, for parent [^{57}Co]-cobalt(II) species, along with the corresponding daughter [^{57}Fe]-iron(II) species, some portion of [^{57}Fe]-iron(III) species can be formed. These after-effects, which inevitably complicate the emission spectra, yet can provide valuable additional information, e.g. on the electron-acceptor properties of the proximal coordination environment of the metal under study [14,15]. For instance, the higher the electron acceptor properties of the microenvironment of the daughter ^{57}Fe atom, the higher the yield of the aliovalent [^{57}Fe]-ferric form.

As the recoil-free emission (as well as absorption) of gamma-quanta (the Mössbauer effect) is observed in solids only, solutions or liquids are usually studied rapidly frozen [8]. Rapid freezing often allows crystallisation of the liquid (solvent) to be avoided, so that the structure of the resulting glassy solid matrix represents that of the solution. Upon freezing, biochemical (metabolic) processes in live cells, tissues or other biological samples cease at a certain point. Thus, for live bacterial cells in contact with $^{57}\text{Co}^{2+}$, freezing after different periods of time allows both the initial rapid binding of the metal cation by cell-surface biopolymers and its further metabolic transformations to be monitored. On the other hand, in cells or tissues kept above the freezing point, any noticeable Mössbauer effect can be detected only for ^{57}Co species within (or at the surface of) quasi-solid parts (e.g. cell membranes) or in a very viscous medium.

The experimental set-up for measuring emission Mössbauer spectra is schematically shown in Fig. 1. The ^{57}Co -containing sample (which in EMS is the source of gamma-radiation) can be kept in a cryostat (e.g. in liquid nitrogen at $T \approx 80$ K), whereas the absorber vibrates along the axis 'source-absorber' at a constant acceleration value (with its sign periodically changing from $+a$ to $-a$) with

a strictly defined time dependence of velocities (usually up to ± 10 mm/s relative to the sample), thus modifying the gamma-quanta energy scale according to the Doppler effect.

EMS measurements are performed by placing a ^{57}Co -containing sample (as a source of γ -radiation) in a sample holder of the spectrometer (if necessary, in a specially designed cryostat with a window for gamma-rays) using a conventional constant-acceleration Mössbauer spectrometer combined with a PC-operated multi-channel analyser. The spectrometer is calibrated using a standard (e.g. α -Fe foil). In order to obtain suitable statistics, each spectrum is collected for some period of time, usually from a few hours up to several days, depending on the content of ^{57}Co in the sample and the Mössbauer effect [8,9]. Standard PC-based statistical analysis consists of fitting the experimental data obtained (for EMS they are commonly converted into a form compatible with that of conventional absorption ^{57}Fe Mössbauer measurements, with isomer shift values positive with regard to α -Fe) as a sum of Lorentzian-shaped lines using a least squares minimisation procedure. The Mössbauer parameters calculated from the experimental data are the isomer shift (IS; relative to α -Fe), quadrupole splitting (QS), linewidth (i.e. experimentally obtained full width at half maximum, FWHM) and relative areas of subspectra (S_r).

3. Experimental results and discussion

3.1. Microbiological applications

Cobalt, being a biologically essential trace element, yet can be toxic for many organisms at higher concentrations [17]. In particular, when present in the medium, Co^{2+} (as well as some other heavy metals) is rapidly accumulated in most bacterial cells by the fast and unspecific membrane-integral protein CorA belonging to the metal-inorganic-transport (MIT) family [1,17]. In many microorganisms, cobalt(II) is involved in diverse enzymatic activities [1–7,17–19] and can be included in intracellular magnetosomes [20]. However, its primary binding to the cell surface in the course of purely chemical and virtually non-selective processes can occur both in live and dead microbial cells [1].

Cobalt attracts attention also owing to biogeochemical problems resulting from the microbially mediated migration of the radionuclide ^{60}Co from disposal sites [21]. Such processes can be facilitated by release of cobalt traces entrapped within ferric oxide minerals upon the microbial

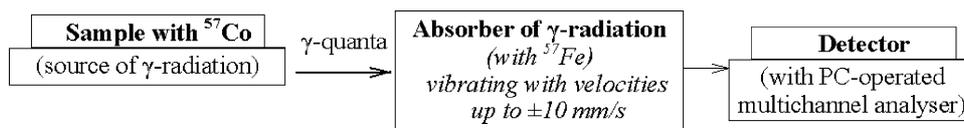


Fig. 1. Scheme of experimental set-up for measuring emission Mössbauer spectra.

dissimilatory reduction of iron(III) [22] together with possible Co^{3+} species [23]. In view of the aforementioned, knowledge of microbiological transformations of cobalt is of importance both from the analytical viewpoint and for its chemical speciation.

Virtually the first report on EMS measurements on bacteria concerned the accumulation of $^{57}\text{Co}^{2+}$ complex with a bacterial hexadentate iron(III)-chelating agent, enterochelin (a cyclic trimer of *N*-2,3-dihydroxybenzoylserine) in *Escherichia coli* (strain AN 272) [24]. Measurements were performed both for frozen bacterial samples (at $T=83\text{ K}$) and above the freezing point (at $+3\text{ }^\circ\text{C}$). It is interesting that in the latter case a very weak but distinguishable effect was detected for 24 h, after which no effect was any longer observed. Disappearance of the effect was ascribed by the authors [24] to damage which might have occurred to the cells under the experimental conditions applied. For frozen bacteria, the effect was much higher and resulted in accumulating good spectra within 4 days. Comparing the data obtained, it was assumed that about 4% of the $^{57}\text{Co}^{2+}$ complex absorbed by the bacteria were located within the cell membrane (a quasi-solid cellular structure), thus giving some weak but noticeable effect without freezing. A similar EMS study was performed on ^{57}Co in cyanobacteria (blue green alga), and the data obtained were compared with absorption (^{57}Fe) Mössbauer measurements on iron species in alga cells [25].

In the soil rhizobacterium *Azospirillum brasilense* (strain Sp245), which had previously been shown to be tolerant to submillimolar concentrations of heavy metals, including cobalt(II), in the culture medium [26–28], EMS studies were first performed on freeze-dried bacterial samples (measured at $T=80\text{ K}$) [18]. The following experiments with the same bacteria were performed with live bacteria rapidly frozen after certain periods of time (2–60 min) of contact with [^{57}Co]-cobalt(II), and EMS spectra were measured for frozen suspensions (without drying), which more closely represents the state of cobalt in the live cells [29] (Fig. 2a). Nevertheless, comparing the data for freeze-dried bacteria [18] and for those measured in frozen aqueous suspensions shows that their Mössbauer parameters are very close (both for 2 min and for 60 min of contact with $^{57}\text{Co}^{\text{II}}$, whereas there were significant differences in the parameters between the two periods). Typical EMS spectra of a rapidly frozen cell suspension and cell-free supernatant liquid shown in Fig. 2a and b also clearly indicate differences between them. Note that there are two forms referring to cobalt(II) represented by quadrupole doublets with larger QS values (whereas the third doublet with smaller IS and QS values corresponds to the aliovalent daughter [^{57}Fe]-ferric form resulting from after-effects). Multiple forms of cobalt(II) found in the spectra are related to the availability of different functional groups (with possibly different donor atoms) as ligands at the cell surface of *Azospirillum* [30,31].

In Fig. 3, the Mössbauer parameters are plotted for different cobalt(II) forms in each sample and for various periods of contact (2 and 60 min) of the live bacteria

with $^{57}\text{Co}^{\text{II}}$, as well as for dead bacterial cells (thermally killed by storing in the medium at $95\text{ }^\circ\text{C}$ in a water bath) and for the cell-free supernatant liquid. Note that the parameters for both the forms of [^{57}Co]-cobalt(II) are statistically significantly different for different periods of contact (2 and 60 min) of live bacteria with the metal. This shows that cobalt(II) rapidly sorbed by the live cells undergoes metabolic transformation within an hour. Nevertheless, the parameters for live bacteria after 2 min and for dead bacteria are rather close (essentially overlapping). This finding indicates that the mechanism of primary rapid Co^{2+} sorption by live cells is similar to the purely chemical binding process occurring at the surface of dead (thermally killed) cells, and is virtually not affected by such hydrothermal treatment. Note also that the parameters for the cell-free supernatant liquid (from which the bacterial cells were removed by centrifugation) are clearly different from those for all the other samples (see Fig. 3).

It should be mentioned that primary binding of heavy metals by the cell surface in Gram-negative bacteria is mediated by capsular polysaccharide (PS, particularly carboxylated acidic PS), lipopolysaccharide (LPS, including phosphate LPS moieties) and proteinaceous materials (see, e.g. [1,17,32] and references therein). In *A. brasilense*, these biopolymers and their covalently bound complexes characteristic of the cell surface [30,31] are believed to be involved in contact interactions with plant roots [30,33,34] and in bacterial cell aggregation [33,35,36]. Note that direct identification of metal complexes in such sophisticated systems as bacterial cells is at the present step difficult, as various chemical species with similar coordination micro-environment can give similar Mössbauer parameters. Also, in order to ascribe the parameters found in the EMS spectra of bacterial cells to certain biocomplexes, model experiments with a range of isolated cell surface biopolymers are needed; such data are not yet available which hampers further detailed interpretation of the spectroscopic changes observed.

In the cell-free culture solution, the most likely ligands which could bind Co^{2+} are phosphate and possibly malate anions (they are present in the growth medium [26–29] at concentrations of the order of 10^{-2} M), ammonia (from NH_4^+ added to the initial medium at $\sim 5\text{ mM}$), as well as various (probably acidic) exopolysaccharides dissolving from the cell surface [30]. Note that both malate and ammonium are gradually consumed by the growing bacteria as sources of carbon and bound nitrogen. Thus, the majority of donor atoms in the first coordination spheres of $^{57}\text{Co}^{2+}$ complexes in the cell-free solution are likely to be represented by oxygen (including that of hydration water molecules) and probably nitrogen. The largest QS value (QS=3.2 mm/s, see Fig. 3) is rather close to that for the ferrous hexaquo complex [8–10]; however, the value of IS=1.22 mm/s (see Fig. 3) for that form is significantly lower. In view of that, the presence of ‘free’

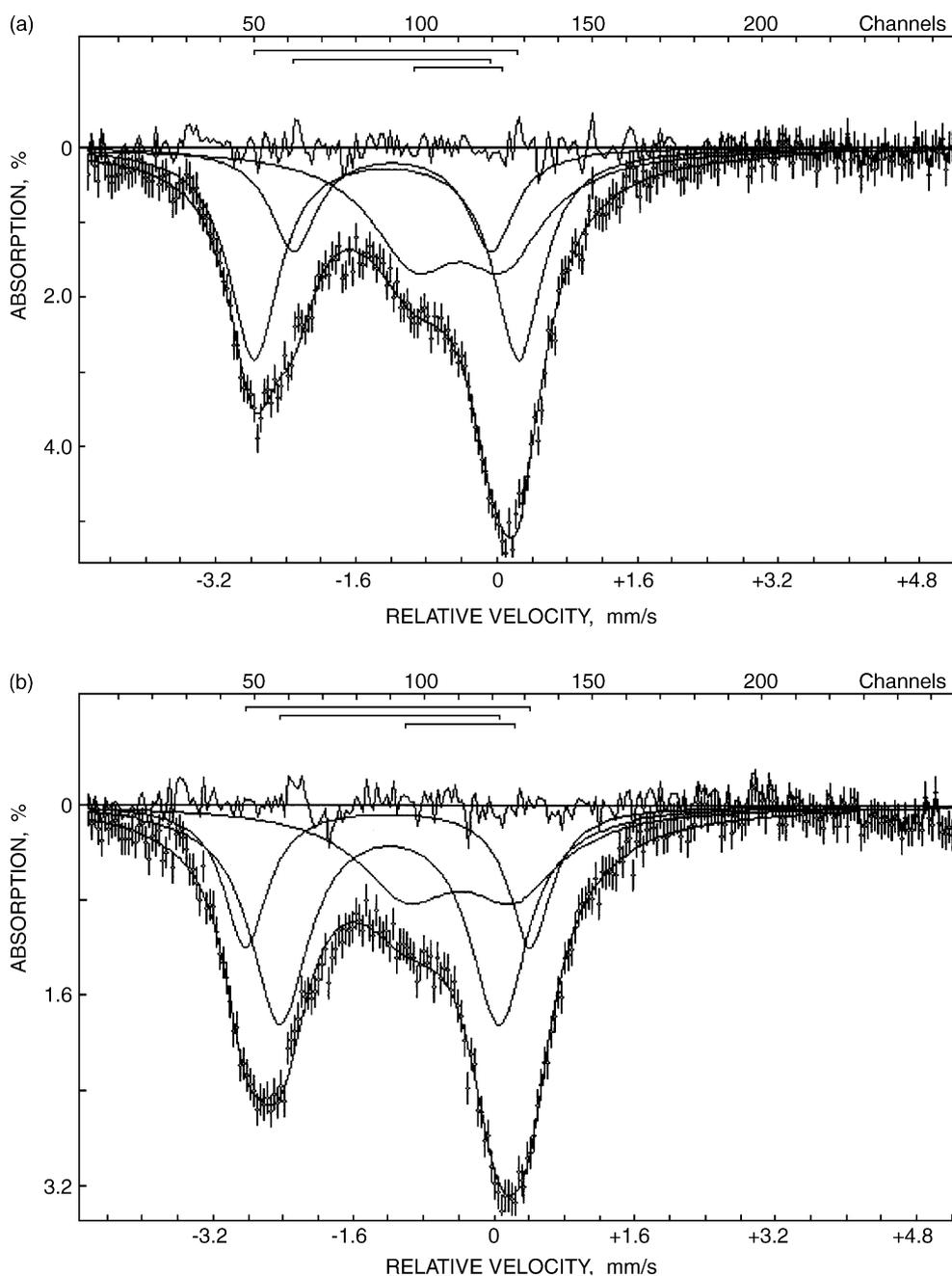


Fig. 2. Typical emission Mössbauer spectra (a) of aqueous suspension of live cells of *Azospirillum brasilense* Sp245 in the culture medium (frozen 2 min after contact with $^{57}\text{Co}^{\text{II}}$) as well as (b) of the cell-free supernatant liquid rapidly frozen in liquid nitrogen (spectra collected at $T=80$ K). For each spectrum, the relevant subspectra are also shown which contributed to the resulting spectrum (solid-line envelope) obtained by computer fitting to the experimental data (points).

$[\text{}^{57}\text{Co}(\text{H}_2\text{O})_6]^{2+}$ cations in the cell-free culture solution in somewhat noticeable amounts is unlikely.

3.2. Biochemical applications

This field of EMS applications is virtually unexplored, though our recent first studies on $[\text{}^{57}\text{Co}]$ -cobalt-containing biocomplexes [18,19] have shown great promise. It has to be noted that a number of EMS studies on $[\text{}^{57}\text{Co}]$ -cobalt-containing coordination compounds with a range of ligands,

including organics, have contributed to understanding methodological and interpretational aspects of the EMS technique applied to diverse problems related to coordination and radiation chemistry. In earlier studies [14,15,37–40], consequences of the nuclear decay in ^{57}Co and heteronuclear (^{57}Co – ^{57}Fe , etc.) complexes in different solid matrices have been investigated. Mixed-valence states have been studied for ^{57}Co -labelled trinuclear Co–Fe carboxylates [41]; in a later comprehensive study [42] it has been found using a comparison of EMS and absorption

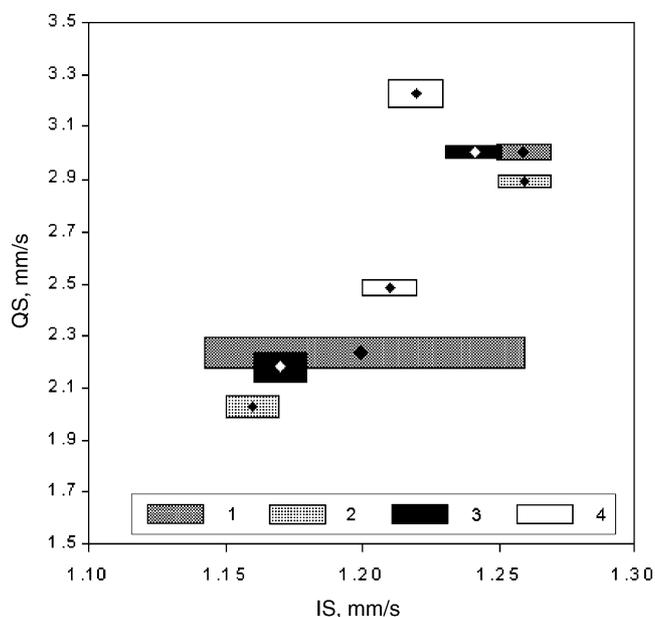


Fig. 3. Comparison of Mössbauer parameters—Isomer Shift (IS, mm/s; relative to α -Fe) and Quadrupole Splitting (QS, mm/s)—for different forms of ^{57}Co -cobalt(II) in aqueous suspension of live cells of *Azospirillum brasilense* Sp245 rapidly frozen after (1) 2 min; (2) 60 min of contact with $^{57}\text{Co}^{\text{II}}$; (3) dead cells (hydrothermally killed at 95 °C); and (4) cell-free supernatant liquid (measured at $T=80$ K).

(^{57}Fe) Mössbauer measurements that no valence delocalisation occurs between different elements (e.g. Co and Fe). It has also been shown on the basis of the data obtained [42] that most of the decayed ^{57}Co atoms (having transformed into ^{57}Fe) retain their positions at the sites of parent ^{57}Co atoms, which is essential for the correct interpretation of experimental EMS data.

Our recent studies on the cation specificity of bacterial glutamine synthetase (GS), which is a key enzyme of nitrogen metabolism ubiquitous in many organisms, have shown that cobalt(II) is one of the main activating cations for the GS, also playing a role in maintaining its secondary structure [4,19]. It was found possible to remove native cations from the enzyme by its EDTA treatment and further dialysis; in that case the enzyme lost its activity without divalent cations in the medium [4]. As the GS activity was found to be restored after adding cobalt(II), it was indicative of the cation entering the GS active centres, which is prerequisite for the activity of the enzyme to be expressed [43]. Thus, incubating the cation-free (EDTA-treated and dialysed) GS with a calculated amount of $^{57}\text{Co}^{2+}$ salt and rapidly freezing the solution, it was possible to obtain its EMS spectra characterising the ^{57}Co -cobalt(II)-bearing active centres of the enzyme [19]. If the amount of $^{57}\text{Co}^{2+}$ (i.e. total cobalt(II) added) is lower than 24 mol per 1 mol of GS (as the bacterial GS molecule, according to the present views [43], contains 12 active centres with two different cation-binding sites each), it might be possible to quantitatively evaluate ^{57}Co -cobalt(II) distribution between the sites.

Analysis of the emission Mössbauer spectra of ^{57}Co -cobalt(II)-doped GS both in the solid state and in rapidly frozen aqueous solution has indeed shown two forms of cobalt(II) with different affinities (in view of non-equal distribution of $^{57}\text{Co}^{\text{II}}$ between the forms [19]) as well as with different coordination reflected by different Mössbauer parameters shown in Fig. 4. However, each of the relevant forms in dissolved (see 1 in Fig. 4) and solid enzyme (see 2 in Fig. 4) has similar (significantly overlapping) parameters. This is indicative of a close similarity of the cobalt(II) coordination structure in enzyme's active centres both in solution and in the dry state, which reflects the well-known fact of stability of the enzyme upon drying.

It should also be mentioned that the parameters (IS and QS; see Fig. 4) allow the cation-binding sites in azospirillum GS to be ascribed to certain types of coordination. It may be assumed that the respective nucleogenic Fe^{II} forms with a lower $\text{QS}=2.3\text{--}2.4$ mm/s (see Fig. 4) correspond to the cation-binding site with more symmetrically coordinated O-donor ligands. Note that, in the case of *Salmonella typhimurium* GS, the cation in site n1 (with a much higher affinity) is coordinated by three glutamate (Glu) residues (i.e. three carboxylic groups), whereas in site n2 by one histidine (His) and two Glu residues (i.e. one N-donor atom of the His heterocycle and two carboxyls), and this structure is conserved among different GSs [43] (additional ligand(s) are one or more H_2O molecules [44]). In this case, for *A. brasilense* GS, the higher QS value (ca. 3.0–3.1 mm/s;

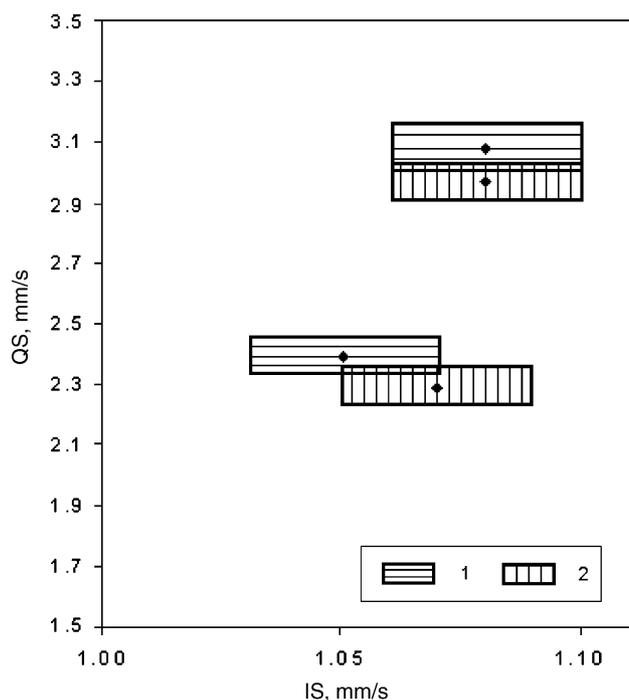


Fig. 4. Comparison of Mössbauer parameters—Isomer Shift (IS, mm/s; relative to α -Fe) and Quadrupole Splitting (QS, mm/s)—for different forms of ^{57}Co -cobalt(II) in $^{57}\text{Co}^{\text{II}}$ -doped glutamine synthetase from *Azospirillum brasilense* Sp245 (1) in rapidly frozen aqueous solution and (2) in the solid (dried) state (measured at $T=80$ K).

see Fig. 4) may reflect a lower coordination symmetry owing to different donor atoms (the IS values are rather close in these cases). Similar Mössbauer parameters were reported for Fe^{II}-DNA complex: IS=1.07 mm/s and QS=2.98 mm/s at 300 K [9], p. 382). Note that the heterocycle of the His residue is similar to the five-membered heterocycle in purine which, in neutral DNA solutions, may coordinate Fe^{II} at the N7 atom. This conclusion is also in line with a noticeably higher proportion of the Fe^{II} form with QS=2.3–2.4 mm/s [19] (see Fig. 4) which in *A. brasilense* GS may thus correspond to the n1 site (with a higher affinity to cations), the other Fe^{II} form corresponding to the n2 site.

4. Conclusions and outlook

EMS measurements in frozen aqueous solutions have shown that the process of primary adsorption of cobalt(II) traces by the bacterial cells is evidently rapid and complete, with its further metabolic transformations occurring within an hour. Comparison of the Mössbauer parameters for live and dead bacterial cells indicates that the processes of primary rapid adsorption of cobalt(II) by live cells are chemically similar to its interaction with hydrothermally killed bacteria. The results obtained demonstrate that EMS is a valuable tool for the monitoring of trace cobalt uptake and its metabolic transformations in complicated biological systems including, e.g. living bacterial cells (in vivo) with ongoing metabolic processes.

In studying the structural organisation of cation-binding sites within the active centres of metalloenzymes, those bearing Co²⁺ (also together with other cations, in particular, in cases of double-site active centres related to two-metal-ion catalysis) can be probed by EMS using ⁵⁷Co²⁺-doped enzyme samples. This allows the chemical state and coordination of cobalt as well as its distribution between the sites to be analysed, as has been illustrated by the data on glutamine synthetase, a ubiquitous key enzyme of nitrogen metabolism, isolated from *A. brasilense*. In this respect, the power of the EMS technique can hardly be overestimated, as measurements of rapidly frozen solutions of enzymes or metalloproteins can be sensitive to fine structural rearrangements at the cation-binding site accompanying, e.g. enzyme-substrate biospecific interactions or covalent modifications of the enzyme molecule. The data obtained might thus give a key to solving otherwise 'inexpugnable' mechanistic problems in molecular biology of metalloproteins.

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