Anal Bioanal Chem (2002) 372 : 431–435
DOI 10.1007/s00216-001-1116-7

SPECIAL ISSUE PAPER

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Trace cobalt speciation in bacteria and at enzymic active sites using emission Mössbauer spectroscopy

Received: 15 May 2001 / Revised: 5 August 2001 / Accepted: 25 August 2001 / Published online: 20 December 2001

Abstract ^{57}Co emission Mössbauer spectroscopy (EMS) allows the chemical state of cobalt, as influenced by its coordination environment, to be monitored in biological samples at its physiological (trace) concentrations. To draw attention to EMS as a valuable tool for speciation of cobalt in biocomplexes, the process of cobalt(II) metabolism in cells of the plant growth-promoting rhizobacterium *Azospirillum brasilense* Sp245 was investigated using EMS of $^{57}Co^{II}$-doped bacterial cells. EMS measurements also showed $^{57}Co^{II}$-activated glutamine synthetase (GS, a key enzyme of nitrogen metabolism, isolated from this bacterium) to have two different cobalt(II) forms at its active sites, in agreement with data available on other bacterial GSs. Chemical after-effects following electron capture by the nucleus of the parent $^{57}Co^{II}$ during the $^{57}Co \rightarrow {}^{57}Fe$ transition, which contribute to the formation of a stabilised daughter $^{57}Fe^{III}$ component along with the nucleogenic $^{57}Fe^{II}$ forms, are also briefly considered.

Keywords Emission Mössbauer spectroscopy · Cobalt (II) · *Azospirillum brasilense* · Bacterial cells · Glutamine synthetase · Enzyme active sites

Introduction

The increasing importance of bioanalysis in the ever changing field of analytical chemistry [1] is largely related to speciation of trace elements, since both beneficial and toxic effects of a particular trace element in biology and medicine can be attributed to its certain chemical species [2]. As reviewed recently by Weise et al. [3], nuclear analytical methods generally do not allow speciation analysis to be performed, because the analytical result is not influenced by the chemical state of the element under quantification. Nevertheless, the emission variant of Mössbauer (nuclear gamma-resonance) spectroscopy (EMS), which is commonly not regarded as a purely analytical method (albeit capable of providing quantitative data on the content of certain chemical species) and is not mentioned in the above review [3], can provide essential information on the atomic level directly on the chemical state of the trace nuclide. This is due to its much higher sensitivity as compared to its absorption variant. The main limitation of the method is the availability of the elements having Mössbauer-active nuclides and the ease of their application [4, 5]. However, if the element is suitable, the information obtained meets the requirements of speciation analysis which can readily be adapted to be performed in situ. In certain cases when not only analytical aspect is of importance, EMS may well be a valuable complement to other methods.

^{57}Co is the most widely used radionuclide for EMS [5]. Its nuclear decay via electron capture results in the formation of ^{57}Fe accompanied by certain physical and chemical after-effects [5, 6]. The resulting recoil energy (ca. 4.6 eV) for the daughter ^{57}Fe nucleus is sufficiently low, and the daughter iron atoms do not shift from their positions [5]. Therefore in many cases their chemical state reflects that of the parent ^{57}Co species. Cobalt is a biologically essential trace element with a broad range of physiological and biochemical functions [7, 8, 9, 10, 11] and can be toxic at high concentrations [12, 13, 14, 15]. It attracts attention also owing to microbially mediated biogeochemical problems related to the migration of the radionuclide ^{60}Co from disposal sites [16, 17]. In view of that, the application of ^{57}Co EMS to studying biological transformations of cobalt is warranted both from the analytical viewpoint and for its chemical speciation.

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Our previous studies on the plant growth-promoting rhizobacterium *Azospirillum brasilense* have shown that 0.2 mmol L^{-1} cobalt(II) in the culture medium taken up by the bacterium can induce some metabolic changes in the latter as a response to metal stress [18, 19]. Co^{2+} was also shown to support the activity of glutamine synthetase (GS, a key enzyme of nitrogen metabolism) from this bacterium [9, 20]. In the present work, Co^{2+} metabolism in *A. brasilense* Sp245 was followed using EMS of bacterial cells (stored in aqueous suspensions with added traces of $^{57}CoCl_2$ and frozen after certain periods of time). EMS was also used to study $^{57}Co^{2+}$-activated GS, after it had been isolated from *A. brasilense* Sp245 and purified.

Experimental

Azospirillum brasilense Sp245 was cultivated in a standard mineral medium as described elsewhere [9, 18, 19, 20], with 5 mmol L^{-1} NH_4Cl as a nitrogen source and 0.6% sodium malate as a carbon source (pH 6.9). For EMS studies of Co^{2+}-treated cells, the bacterial culture was grown up to ca. 2.4×10^8 cells mL^{-1} (by monitoring the optical density of the suspension), that corresponds to the mid-exponential growth phase, and stored for 1 day at 4 °C. Then 1-mL aliquots of the cell suspension were incubated at room temperature for 1 h, placed into PTFE sample holders each containing 1 mCi of radioactive $^{57}CoCl_2$ free from natural Co^{II}, i.e. "carrier-free" (obtained from the Centre for Radionuclide Diagnostics, Moscow State University, Russia), that had been dried from aqueous solution (final $^{57}Co^{2+}$ concentration ca. 2 μmol L^{-1}), closed to prevent evaporation, and after 2 and 60 min the corresponding samples were rapidly frozen in liquid nitrogen. Before EMS measurements, the intact cell samples were freeze-dried to enhance the Mössbauer effect (intensity of the spectra), which appeared to be weak in frozen aqueous suspensions (this could be due to different recoilless absorption probabilities, reflected by the Debye–Waller factor [5], for a dried solid and a glass-like matrix of a frozen solution).

To isolate GS, *A. brasilense* Sp245 cells were disrupted by passing them through a French press [20]. They were then treated with 0.25 mg mL^{-1} DNAse I (Sigma) and 2 mg mL^{-1} streptomycin sulfate, centrifuged, and the supernatant was subjected, first, to ion-exchange chromatography in 50 mmol L^{-1} Tris-HCl buffer (pH 7.0) with a linear NaCl gradient from 0 to 1 mol L^{-1} using DEAE-Toyopearl 650 M (Toyo Soda), and then to FPLC on a Mono-Q column (Pharmacia Biotech) with the same buffer and NaCl gradient. The homogeneous GS preparation obtained was treated for 30 min with 5 mmol L^{-1} EDTA (to remove metal cations, bound to native GS [20], from the enzyme) and then dialysed against 1000 volumes of 50 mmol L^{-1} KCl (pH 7.0) at 4 °C to separate the enzyme from EDTA–metal chelates. Carrier-free $^{57}CoCl_2$ (1 mCi) was added to 1 mL of aqueous solution of the resulting cation-free enzyme up to a ratio of ca. 24 $^{57}Co^{2+}$ ions per enzyme molecule (see below). This solution was then incubated for 1 h at ambient temperature, and finally rapidly frozen in liquid nitrogen.

EMS measurements were performed by placing the ^{57}Co-containing sample (source) in a cryostat filled with liquid nitrogen (at ca. 80 K) using a conventional constant-acceleration Mössbauer spectrometer (absorber $K_4[Fe(CN)_6].3H_2O$) combined with a PC-operated multichannel analyser. Standard PC-based statistical analysis consisted of fitting the experimental data obtained (converted into a form compatible with that of absorption ^{57}Fe Mössbauer measurements) as the sum of Lorentzians using a least-squares minimisation procedure, which enabled determination of the isomer shift (IS; relative to α-Fe), quadrupole splitting (QS), linewidth (i.e. full width at half maximum, FWHM), and relative areas of spectral components (S_r).

Results and discussion

A. brasilense Sp245 cells

Binding of heavy metals by the cell surface in Gram-negative bacteria is mediated primarily by capsular polysaccharide (PS) and lipopolysaccharide (LPS) materials [21, 22]. The latter are characteristic for *A. brasilense* as well and, along with its outer-membrane proteins, are believed to be involved in contact interactions with plant roots and in bacterial cell aggregation [23, 24, 25].

In the case of a relatively high uptake level of cobalt(II) in *A. brasilense* [18, 19], it might well be expected that traces of $^{57}Co^{2+}$ would be completely bound by its cell surface. This is corroborated by the EMS data (Fig. 1a; Table 1, sample 1). Indeed, after only 2 min of contact with bacterial cell suspension, the spectrum at 80 K exhibits two high-spin components [26] corresponding to oxidation state +2, neither of which, however, corresponds to $[^{57}Co(H_2O)_6]^{2+}$ that has QS>3.2 mm s^{-1} [5] (our similar measurements in the cell-free supernatant showed ca. 16% of all the $^{57}Co^{II}$ present as the hexaaquo complex). Thus, the process of cobalt absorption by the bacte-

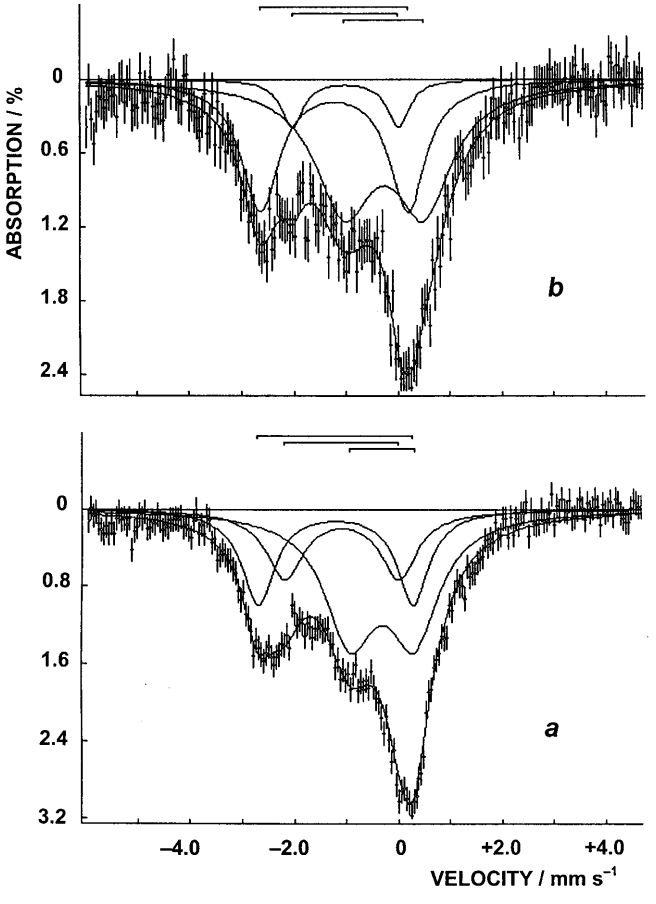

Fig. 1 Emission Mössbauer spectra of *Azospirillum brasilense* Sp245 living cells frozen (**a**) 2 min and (**b**) 1 h after incubation with trace $^{57}CoCl_2$ (dried; T=80 K)

Table 1 Mössbauer parameters[a] calculated from emission Mössbauer spectra of $^{57}Co^{II}$-containing cells of *Azospirillum brasilense* Sp245 and $^{57}Co^{II}$-activated glutamine synthetase isolated from this bacterium (T=80 K)

Sample	Oxidation state[b]	IS[c] (mm s^{-1})	QS[d] (mm s^{-1})	FWHM[e] (mm s^{-1})	S_r[f] (%)	Fig.
1. Bacterial cells (frozen 2 min after adding $^{57}CoCl_2$)	+2	1.24 (3)	3.08 (6)	0.70(10)	19(1)	1a
	+2	1.14 (3)	2.35 (9)	0.83(13)	23(1)	
	+3	0.35 (5)	1.26 (8)	1.43(12)	58(1)	
2. Bacterial cells (frozen 60 min after adding $^{57}CoCl_2$)	+2	1.22 (4)	2.84 (7)	0.88(10)	38(1)	1b
	+2	1.00 (5)	2.03 (9)	0.52(25)	8(1)	
	+3	0.26 (5)	1.55 (7)	1.36(16)	54(1)	
3. Glutamine synthetase (cation-free enzyme solution frozen 60 min after adding $^{57}CoCl_2$)	+2	1.08 (2)	3.08 (8)	0.48 (5)	18(1)	2
	+2	1.05 (2)	2.39 (6)	0.75 (8)	60(1)	
	+3	0.34(10)	1.12(20)	1.25(30)	22(1)	

[a]Errors (in the last digits) are given in parentheses
[b]Oxidation states of the daughter ^{57}Fe components stabilised after nuclear decay of the parent $^{57}Co^{II}$
[c]Isomer shift (relative to α-Fe) converted to the normal absorption convention (positive with regard to α-Fe)
[d]Quadrupole splitting
[e]Full line width at half maximum
[f]Relative resonant absorption areas of the relevant spectral components, which represent relative contents of the corresponding Fe forms assuming a common recoilless fraction for all forms in a sample

rial cells is evidently rapid and complete. It should be noted, however, that the yield of stabilised daughter $^{57}Fe^{III}$ component as a consequence of after-effects [5] (58%, see Table 1, sample 1) is similar to the 57% observed in very dilute, rapidly frozen (80 K) aqueous solutions of $^{57}CoCl_2$ [5]. (Owing to insufficient resolution of the lines of the daughter $^{57}Fe^{III}$, its contribution was approximated by one doublet, which does not influence the resolution of the $^{57}Fe^{II}$ lines; see Table 1). Thus, Co^{II} initially bound to bacterial cell surface biopolymers is likely to contain coordinated water, or, as an alternative explanation, the ligands must have similar relatively high electron-acceptor properties and/or be similarly prone to localised self-radiolysis [6, 27, 28].

The former reason seems more likely considering the higher hydration of heavy metal-stressed *A. brasilense* cells (including Co^{II} taken up by the bacterium) [19]; it has been previously noted that some heavy metals bound to LPS phosphate groups can induce their hydration [29]. The presence of two Co^{II} forms (with different IS and QS; see Table 1, sample 1) may be connected with the availability of different ligands at the cell surface [18, 19, 25, 26, 27]. It is interesting to note that, after 1 h of incubation of live cells with $^{57}Co^{II}$, the Mössbauer parameters changed (cf. Figs 1a and 1b; samples 1 and 2 in Table 1). The observed decrease in QS for the daughter $^{57}Fe^{II}$ component with IS=1.22–1.24 mm s^{-1} and its doubled proportion (38%) in sample 2, as well as the appearance of the $^{57}Fe^{II}$ component with lower IS (1.00 mm s^{-1}) and QS (2.03 mm s^{-1}) indicates further metabolism of $^{57}Co^{II}$, following its primary absorption. This could arise from the formation of species with a lower coordination number (with O-donor ligands), as well as with more covalent coordination bonds (involving, e.g. N-donor atoms) for the latter component [5, 26]. This may also account for a slight decrease in the yield of the stabilised daughter $^{57}Fe^{III}$ component in sample 2. Note for comparison, that EMS studies in *Escherichia coli* showed ^{57}Co, taken up from enterocholine solution, to reside within the cell membrane for 24 h, after which it was found in the interior of the cell ([26], p. 386). In view of that, it may be inferred that the observed changes reflecting cobalt(II) metabolism in *A. brasilense* Sp245 within an hour (see Fig. 1a,b and Table 1, samples 1,2) are localised within the bacterial cell wall containing PS-, LPS- and protein-containing moieties typical for azospirilla [19, 23, 24, 25].

^{57}Co-activated glutamine synthetase from *A. brasilense* Sp245

In bacteria, GS plays a key role in nitrogen metabolism; most well studied are GSs from enteric bacteria only [30]. Bacterial GSs are dodecameric proteins; the GS molecule is formed from two face-to-face hexameric rings of total 12 subunits with 12 active sites between the monomers, each having two divalent cation-binding sites, n1 and n2, which differ in their affinity for divalent cations [31]. For all GSs, including that of *A. brasilense*, divalent cations (commonly, Mg^{2+}, Mn^{2+} or Co^{2+}) are necessary for the activity to be expressed [9, 20, 30, 31, 32]. Along with the twelve n1 sites and twelve n2 sites, GS from *E. coli* has 48 additional metal-binding sites per oligomer with relatively lower affinity [32]. In our opinion, this is likely to be the case also for *A. brasilense* GS. In view of that, considering also the possibility for higher concentrations of Co^{2+} (and Zn^{2+}) to induce GS polymerisation [33, 34], in order to avoid multiple-site binding of excessive $^{57}Co^{2+}$ ions which would lead to highly complicated spectra, in our experiment we applied a ratio of up to 24 $^{57}Co^{2+}$ ions per GS molecule in solution.

The resulting spectrum of cation-free *A. brasilense* GS incubated for 1 h with $^{57}Co^{2+}$ in rapidly frozen solution (Fig. 2) contains two components corresponding to two

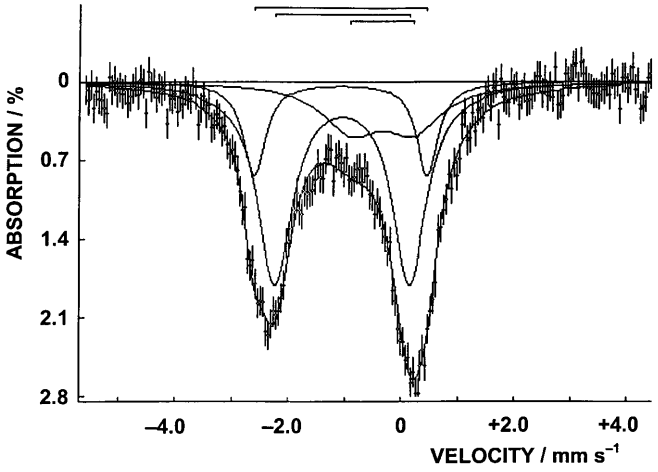

Fig. 2 Emission Mössbauer spectrum of cation-free glutamine synthetase from *Azospirillum brasilense* Sp245 activated by $^{57}Co^{2+}$ (frozen solution; T=80 K)

high-spin daughter Fe^{II} forms (Table 1, sample 3) with different coordination [35]. It may be supposed that the Fe^{II} form with the lower QS (2.39 mm s^{-1}) corresponds to the site with more symmetrically coordinated O-ligands. Note that, in case of *Salmonella typhimurium* GS, the cation in site n1 (with a much higher affinity) is coordinated by three glutamine (Glu) residues (i.e. three carboxyl 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) [30, 31]. If such is the case with *A. brasilense* GS, the higher QS value (3.08 mm s^{-1}) may reflect a lower coordination symmetry owing to different donor atoms (the IS values are close in these cases). Similar parameters were reported for the Fe^{II}–DNA complex: IS=1.07 mm s^{-1} and QS=2.98 mm s^{-1} at 300 K ([26], p. 382); note that in going from 300 K to 80 K, most QS values for ferrous compounds tend to increase slightly [35]. In neutral DNA solutions, purine nitrogen atoms may be involved in coordination with Fe^{II} [26], and it is noteworthy that the heterocycle of the His residue is similar to the five-member heterocycle in purine. This conclusion is also in line with a noticeably higher proportion (60%) of the Fe^{II} component with QS=2.39 mm s^{-1} which may thus correspond to the site of the type n1 (with a higher affinity to cations) in *A. brasilense* GS.

Note also that the yield of the stabilised daughter $^{57}Fe^{III}$ component resulting from after-effects is much lower (22%) for sample 3 (see Table 1 and Fig. 2). This corresponds to firmer binding of parent $^{57}Co^{II}$ to the aforementioned bioligands with higher electron-donor properties in the inter-subunit spaces of the two GS hexamers [30].

Conclusions

EMS measurements have shown that living *A. brasilense* Sp245 cells rapidly and completely absorb traces of $^{57}Co^{2+}$ from the medium before its further metabolism. $^{57}Co^{II}$-activated GS from this bacterium has been found to have two different Co^{II} forms at its active sites with different affinity, in conformity with the data available on GSs from enteric bacteria. The results obtained demonstrate that EMS is a valuable tool for trace cobalt speciation in situ in complicated biological systems including, e.g. living bacterial cells (in vivo) with ongoing metabolic processes.

Acknowledgements The authors are grateful to Prof. G. Zanetti and Dr. M.A. Vanoni (Milan University, Italy), Prof. A. Vértes and Prof. E. Kuzmann (Eötvös University, Budapest, Hungary; collaboration under the Agreement between the Russian and Hungarian Academies of Sciences for 1999–2001), Prof. M.G. Polissiou and Dr. P.A. Tarantilis (Agricultural University of Athens, Greece), Dr. P.H.E. Gardiner (Sheffield Hallam University, Sheffield, U.K.), A.A. Saprykin (Moscow State University, Russia) and Dr. V.A. Bespalov (IBPPM RAS, Saratov, Russia) for helpful assistance and many stimulating discussions, as well as to an anonymous referee for detailed, constructive and helpful comments on the manuscript. This work was supported in part by the EC (INTAS Project 96–1015), NATO (Collaborative Linkage Grant LST.CLG.977664) and by grant No. 205 from the Russian Academy of Sciences' Commission for the Work with Young Scientists under the 6th Competition-Expertise of research projects (for the team at IBPPM RAS, Saratov, Russia). O.B.S. acknowledges support by INTAS Young Scientists Fellowship YSF 00–160. A.A.K. also gratefully appreciates support from Deutsche Forschungsgemeinschaft (DFG, Germany) and the Organising Committee of the Second International Conference on Trace Element Speciation in Biomedical, Nutritional and Environmental Sciences (Munich–Neuherberg, Germany, May 7–10, 2001; special thanks to Dr. B. Michalke, Neuherberg) for his participation in the Conference where this material was presented.

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