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Application of ^{57}Co emission Mössbauer spectroscopy to studying biocomplexes in frozen solutions

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Abstract Emission Mössbauer spectroscopy with the ^{57}Co isotope was used to study very dilute rapidly frozen aqueous solutions of cobalt(II) complexes with low-molecular-weight biomolecules (aromatic amino acids – anthranilic acid and L-tryptophan) and within a sophisticated biopolymer, bacterial glutamine synthetase, a key enzyme of nitrogen metabolism. The appearance of after-effects of the ^{57}Co→^{57}Fe nuclear transformation as well as the coordination properties of the cation and the ligands in the complexes are discussed on the basis of their Mössbauer parameters.

Key words cobalt(II) complexes · frozen aqueous solutions · anthranilic acid · L-tryptophan · metalloenzymes · after-effects · ^{57}Co emission Mössbauer spectroscopy

1 Introduction

In emission Mössbauer spectroscopy (EMS), ^{57}Co is the most widely used radionuclide. The ^{57}Co EMS technique is several orders of magnitude more sensitive than its ^{57}Fe absorption counterpart [1] that, nevertheless, has been widely applied in biology and biomedicine (for recent reports and reviews see, e.g., [2–6]). As for EMS, the obvious methodological difficulties, arising due to using the radionuclide in the samples under study, restrain EMS applications in bioscience [7]. Cobalt has a broad range of physiological and biochemical

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functions; e.g., in many organisms it is involved in diverse enzymatic activities. Therefore its biochemical speciation is of importance but presents a challenge, as the participation of cobalt in physiological processes is limited by its trace concentrations.

In this report, some examples are discussed illustrating the applicability of EMS in studying various biocomplexes in rapidly frozen aqueous solutions, as well as the unique information which can thus be obtained non-destructively in situ. As the Mössbauer effect is observed in a solid matrix only, solutions or liquid samples are commonly studied rapidly frozen [1], so that the overall structure of the resulting frozen (glassy) matrix would adequately reflect that of the initial liquid. The appearance of after-effects of the $^{57}Co \rightarrow ^{57}Fe$ nuclear transformation, though complicating the EMS spectra, yet can provide additional information related to the electron acceptor properties of the ^{57}Co microenvironment [7, 8].

2 Materials and methods

Complexes of [^{57}Co]-cobalt(II) with anthranilic (*o*-aminobenzoic) acid and L-tryptophan ("Serva") were prepared using carrier-free $^{57}CoCl_2$ (i.e., without natural Co^{2+} salt). An aliquot of its aqueous solution (1 mCi) was put into a PTFE sample holder, completely dried in air at about 40°C, and 1 ml of 0.01 M aqueous solution of an amino acid was added to the resulting solid remainder (thus giving over three orders of magnitude in molar excess to $^{57}Co^{2+}$). The solution was stirred, closed to prevent evaporation, after 1 h rapidly frozen in liquid nitrogen and used for EMS measurements.

Preparation of Co^{2+}-containing samples of glutamine synthetase (GS), a ubiquitous key enzyme of nitrogen metabolism [9] isolated from the nitrogen-fixing plant-associated rhizobacterium *Azospirillum brasilense* (strain Sp245), was described in detail elsewhere [10, 11]. Treatment of GS with 5 mM EDTA for 30 min with further dialysis led to the removal of native cations from the enzyme (with subsequent loss of its activity in the absence of divalent cations in the medium). Further incubation of the resulting cation-free GS with Co^{2+} salt restored its activity, thus indicating that Co^{2+} cations enter the GS active centres (total 12 active centres per GS molecule, with *two* cation-binding sites each [9]), which is necessary for the activity of the enzyme to be expressed [9, 10]. Basing on this, the incubation of cation-free GS with a pre-calculated amount of $^{57}Co^{2+}$ salt gave the $^{57}Co^{II}$-doped biocomplex used for EMS measurements.

Emission Mössbauer spectra were collected using a conventional constant-acceleration spectrometer combined with a PC-operated multi-channel analyser and calibrated using α-Fe foil. Samples, which in EMS are sources of γ-radiation, were kept in a specially designed cryostat (with a window for γ-rays) filled with liquid nitrogen (at ca. 80 K). The absorber ($K_4[^{57}Fe(CN)_6]\ 3H_2O$) was attached to a vibrator in the γ-ray beam path (sample–absorber–detector). Other details of EMS measurements and statistical treatment of emission spectra were described elsewhere [7, 12]. All isomer shift values are presented relative to α-Fe at ambient temperature (converted into a form compatible with that of conventional absorption ^{57}Fe Mössbauer measurements, positive with regard to α-Fe).

3 Results and discussion

3.1 Amino acid complexes

Anthranilic (*o*-aminobenzoic) acid (AA) and L-tryptophan (Trp) are biologically important amino acids. They also occur in soil as a result of microbial or plant-root activity, or among

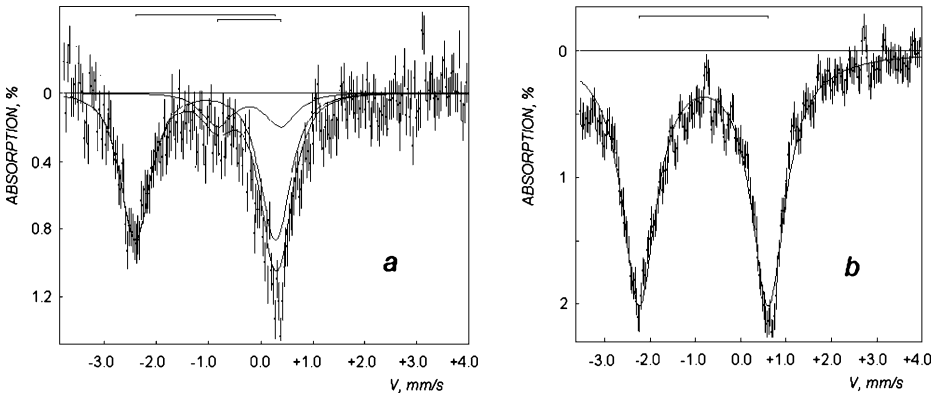

Figure 1 Emission Mössbauer spectra of [^{57}Co]-cobalt(II) complexes with anthranilic acid (a) and L-tryptophan (b) in frozen aqueous solutions (T=80 K).

Table I Mössbauer parameters of emission spectra of ^{57}Co biocomplexes in frozen aqueous solutions (T= 80 K)

Biomolecule	Oxidation state[a]	δ, mm/s	Δ, mm/s	Γ_{exp}, mm/s	S_r, %
AA	+2	1.10(2)	2.71(5)	0.62(4)	75(1)
	+3	0.32(14)	1.1(2)	0.8(2)	25(1)
Trp	+2	0.88(6)	2.84(13)	0.92(1)	100(1)
GS	+2	1.08(2)	3.08(8)	0.48(5)	18(1)
	+2	1.05(2)	2.39(6)	0.75(8)	60(1)
	+3	0.34(10)	1.12(20)	1.25(30)	22(1)

δ, isomer shift (vs. α-Fe at room temperature; converted to the absorption convention); Δ, quadrupole splitting; Γ_{exp}, experimentally observed line width at half maximum; S_r, the relative area of a subspectrum (representing the relative content of the related form, assuming a common recoilless fraction for all forms in a sample).

Errors (in the last digits) are indicated in parentheses.

AA, anthranilic acid; Trp, L-tryptophan; GS, glutamine synthetase (from *A. brasilense*).

[a] For the stabilised nucleogenic ^{57}Fe form.

decay products of proteins (Trp). Thus their interactions with metal ions, including complexation and possible redox transformations [13, 14], are of interest.

Emission Mössbauer spectra of AA and Trp complexes with [^{57}Co]-cobalt(II) (Figure 1a, b) show the presence (Figure 1a) and absence (Figure 1b) of a second doublet of a (+3)-form with a smaller isomer shift and quadrupole splitting (see also Table I). Note that under the conditions applied, no oxidation of Co^{2+} to the (+3) state can take place, so that the (+3) component in AA complex is ascribed to the aliovalent form resulting from after-effects.

The absence of any signs of after-effects, which for $^{57}Co^{2+}$-containing species usually show up as a (+3) component, in the $^{57}Co^{II}$–Trp system is relatively unusual. Considering the possibility for both AA and Trp to chelate Co^{2+} in a similar way (involving the carboxylate and the amino group), the difference in exhibiting after-effects (i.e., in electron-acceptor properties of the metal microenvironment) is probably related to the nature and/or degree of "isolation" of the aromatic moiety. In AA, the benzene ring (which could act as

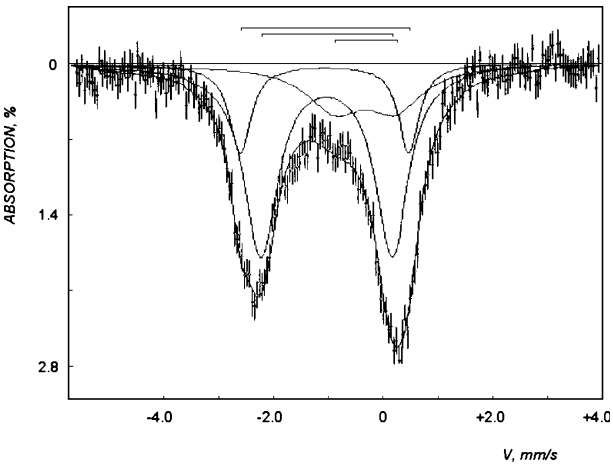

Figure 2 Emission Mössbauer spectrum of $^{57}Co^{2+}$-doped glutamine synthetase from *A. brasilense* in frozen aqueous solution (T=80 K).

an electron acceptor for Auger electrons emitted in the Auger cascade [8]) is closer to the central ion, whereas in Trp it is isolated by an additional >CH–CH_2– moiety.

Furthermore, for the ^{57}Co(II) anthranilate complex, the δ value at T=80 K (δ=1.10 mm/s; see Table I) is noticeably lower than for solid Fe(II) anthranilate [$Fe(C_6H_4(NH_2)CO_2)_2$] (δ= 1.25 mm/s at T=80 K [14]), for which an octahedral coordination has been assumed based on FTIR and ^{57}Fe transmission Mössbauer data (with a bidentate coordination of each of the two COO^- groups, besides the two coordinated amino groups) [14]. Note that the $^{57}Co \rightarrow {}^{57}Fe$ nuclear transformation, proceeding via electron capture by the ^{57}Co nucleus (which thereby turns into ^{57}Fe) with a subsequent Auger cascade developing within 10^{-14} to 10^{-15} s, is followed by emission of a 14.4 keV γ-quantum (ca. 10^{-7} s after the electron capture) [1, 7, 8]. Thus, the resulting substance under investigation using the EMS technique may be described as an ^{57}Fe complex substituted for the 'parent' ^{57}Co binding site (retaining its geometry). Therefore the above mentioned lower δ value found for the $^{57}Co^{II}$–AA complex may indicate that Co^{2+} is coordinated tetrahedrally in complex with AA, in contrast to Fe^{II}–AA complex. The same holds for $^{57}Co^{II}$ complex with Trp, where the δ value at T=80 K is even lower (δ=0.88 mm/s; see Table I). Note that tetrahedral coordination (T_d) is relatively common for cobalt(II) complexes, including biomolecules [15], along with the octahedral symmetry (O_h).

3.2 Metalloenzyme

The structural organisation of enzymic cation-binding sites (bearing Co^{2+} alone or together with other cations, as in cases of double-site active centres related to two-metal-ion catalysis [16]) can also be probed by EMS using $^{57}Co^{2+}$-doped enzyme samples. Thus the coordination of Co and its distribution between the sites can be analysed. This is illustrated by EMS data on $^{57}Co^{2+}$-doped glutamine synthetase isolated from *A. brasilense* [10, 11] (Figure 2; see also Table I).

Note that bacterial GS molecules are dodecamers formed from two face-to-face hexameric rings of subunits, with 12 active centres between monomers, each of the active centres having two divalent cation-binding sites separated by 6 Å, with different coordination and affinity for cations [9]. Earlier we have shown that, along with Mg^{2+}

and Mn^{2+}, cobalt(II) is one of the main activating cations for the *A. brasilense* GS, also playing a role in maintaining its secondary structure [10, 11].

According to Figure 2 and Table I, there are two forms of cobalt(II) in the sample represented two doublets corresponding to high-spin nucleogenic Fe^{2+} (the Fe^{+3} form results from after-effects). The two different Δ values reflect a higher symmetry (the lower Δ) and a lower symmetry (the higher Δ) at each of the two microenvironments of the cation. This is in good agreement with the existence of two separate cation-binding sites at each of the 12 active centres of the GS molecule, viz. a more symmetric site (with three Glu residues as ligands) and a less symmetric site with a significantly lower affinity to the cation (with one His and two Glu residues as ligands) [9–11]. In line with this conclusion, the former site (with a higher affinity for cations and $\Delta \approx 2.4$ mm/s) may be expected to be primarily saturated with $^{57}Co^{2+}$ (featured by a higher S_r value), whereas the latter site (with $\Delta \approx 3.1$ mm/s) binds the remaining $^{57}Co^{2+}$ characterised by a lower S_r value (see Table I).

As in the case with AA and Trp, the relatively low δ values for both the (+2) forms in GS ($\delta = 1.05$ and 1.08 mm/s at $T = 80$ K) may indicate a tetrahedral symmetry of Co^{2+} (in this case, the coordination of all the Glu residues has to be monodentate, which is often observed for cation-binding sites in metalloproteins [15]; note that there is also at least one water molecule as a ligand, according to Eads et al. [17]). As mentioned above, tetrahedral coordination (T_d) of cobalt(II) is possible [15, 18, 19], though in many proteins cobalt was found to have preference for higher coordination numbers, i.e., 5 and 6 (see [19] and references cited therein).

4 Conclusions

Highly sensitive ^{57}Co emission Mössbauer spectroscopy is a valuable tool for structural investigations of biocomplexes with cobalt cations, including their aqueous solutions. Rapid freezing allows one to obtain emission spectra which represent the coordination state of the cation in solution at very low concentrations, as illustrated by the data on amino acid complexes and a metalloenzyme (bacterial glutamine synthetase) doped with $^{57}Co^{2+}$.

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