

## **Co-protected amino acid complexes covalently grafted onto solid supports – syntheses, structural characterization and testing of catalytic activity**

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### **Abstract**

In this work the syntheses of covalently grafted N- or C-protected Co(II)-amino acid (L-histidine, L-cysteine and L-cystine) complexes either with uniform or mixed amino acids ligands are described using chloropropylated silica gel or Merrifield's resin as support. Conditions of the syntheses were altered and the obtained substances were studied by infrared spectroscopy. It was found that in many cases the structures obtained and the coordinating groups substantially varied upon changing the conditions of the syntheses. All the covalently anchored materials displayed superoxide dismutase (SOD) activity. Best of all was found to be the Co-C-protected cysteine covalently grafted onto Merrifield's resin.

### **Introduction**

Homogeneous catalysts are most often complexes of a metal or metal ion and various organic compounds. They can be very active and, occasionally, extremely selective, however, during work-up their separation from the reaction mixture is difficult, therefore, their recovery and reuse seldom can be solved efficiently.

Metal ions are cofactors in many enzymes, most frequently in oxidoreductases. The ions there, are capable of altering their redox states as well as their coordination number. The ligands are amino acid residues of various kinds. Among them one always can find one or more histidine residues. The cofactor together with the proteomic skeleton forms the most selective of all catalysts, the enzymes. They are semi-solid materials capable of working under relatively mild conditions: at near atmospheric pressure, in a limited temperature range and physiological aqueous solution. However, if we prepare metal ion-amino acid complexes inspired by the metal ion containing enzymes, and immobilize them on various supports, then, we may be able to produce catalysts with activities and selectivities resembling those of the enzymes, widening the range of conditions under which the heterogenized complexes are capable of working and at the same time to facilitate easy recovery and recycling.

In order to approach these goals Co-N- or C-protected amino acid (histidine, cysteine, and cystine) complexes were covalently grafted onto rigid or flexible supports, structurally characterized and catalytic activities were tested. Our previous

experiences with the successful synthesis of silica gel grafted Co–mixed ligand N-protected histidine and tyrosine [1] were encouraging.

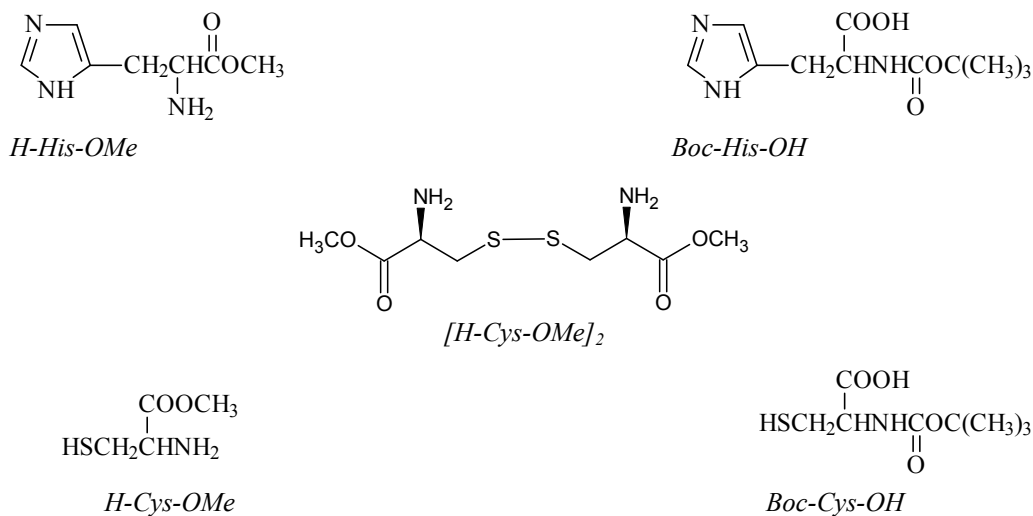
Results of this experimental work are described in the followings.

## Experimental

### *Materials and methods of synthesis*

For the synthesis C- and N-protected L-histidine, L-cysteine and L-cystine,  $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , chloropropylated silica gel (particle size: 230–400 mesh, BET surface area:  $500 \text{ m}^2/\text{g}$ , functionalisation: 8%) or Merrifield's resin (chlorinated polystyrene resin [poly(styrene-co-vinylbenzyl chloride-co-divinylbenzene)] – PS– $\text{PhCH}_2\text{Cl}$  with 3.8–4.2 mmol/g chlorine content) were used. These materials as well as the 2-propanol solvent were the products of Aldrich Chemical Co.

The amino acids (Fig. 1) were covalently grafted onto the modified silica gel or resin surface with esterification (N-protected amino acids) or N-alkylation (C-protected amino acids) like reactions (24 h reflux in a basic suspension). Then, the complexes were built with soaking the amino acid grafted supports in an aqueous solution of the Co salt (24 h stirring at room temperature). These were the complexes made under ligand-poor conditions. In another set of experiments the complexes made were allowed to rearrange in the presence of excess added amino acids (24 h stirring at room temperature) – these were the complexes constructed under ligand-excess conditions.



**Fig. 1.** The C- and N-protected amino acids used in this work (OMe and Boc mean methoxy and tert-butoxycarbonyl groups, respectively)

Surface-grafted complexes were prepared having uniform as well as mixed amino acids (two amino acids were used) as ligands. When mixed amino acids were the ligands two methods were applied for synthesis.

In method A one of the protected amino acids were covalently anchored to the surface of the support, then, it was soaked in the Co salt solution, and after filtering and thorough washing the final substance was made by allowing complexation with excess amounts of the other amino acid.

In method *B* a 1:1 molar mixture of the protected amino acids was grafted to the surface of the support, then, the Co complex was formed (complexation under ligand-poor conditions). Parts of the materials thus formed were further treated in excess 1:1 amino acid mixtures resulting in the formation of surface-anchored complexes under ligand-excess conditions. The experimental conditions were as described above. The protective groups were not removed even after the synthesis, since our previous experiences showed that surface complexes with protective groups were more active than those having deprotected amino acids [2].

#### *Structural characterization by FT-IR spectroscopy*

Structural information on each step of the synthesis procedure was obtained by mid-range infrared spectroscopy, measuring diffuse reflectance. The 3800–600  $\text{cm}^{-1}$  wavenumber range was investigated. Spectra were recorded with a BIO-RAD Digilab Division FTS-65 A/896 FT-IR spectrophotometer with 4  $\text{cm}^{-1}$  resolution. For a spectrum 256 scans were collected. Spectra were evaluated by the Win-IR package. The spectra were treated thoroughly, they were baseline-corrected, smoothed (if it was necessary), the spectra of supports were subtracted and the resulting spectra were deconvolved. In the Figs the more informative 1850–600  $\text{cm}^{-1}$  region is depicted.

#### *Testing the catalytic activity*

Catalytic (SOD) activity was tested by the Beauchamp-Fridovich reaction [3]. For this biochemical test reaction riboflavin, L-methionine and nitro blue tetrazolium were used. Under aerobic conditions reaction takes place on illumination between riboflavin and L-methionine. It is a reduction and the reduced form of riboflavin reacts with oxygen forming a peroxide derivative. This derivative decomposes giving the superoxide radical anion. This radical ion is captured by the nitro blue tetrazolium (NBT) and its original yellow colour turns blue.

The transformation can be followed by spectrophotometry, measuring the absorbance at 560 nm. If our enzyme mimicking material works well, it competes with NBT with success capturing the superoxide radical ion. Thus, the photoreduction of NBT is inhibited. The SOD probe reaction was carried out at room temperature in a suspension of the immobilized complex at pH=7 ensured with a phosphate buffer. The reaction mixture contained 0.1  $\text{cm}^3$  of 0.2  $\text{mmol}/\text{dm}^3$  riboflavin, 0.1  $\text{cm}^3$  of 5  $\text{mmol}/\text{dm}^3$  NBT, 2.8  $\text{cm}^3$  of 50  $\text{mmol}/\text{dm}^3$  phosphate buffer ( $\text{Na}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$ ) containing EDTA (0.1  $\text{mmol}/\text{dm}^3$ ), L-methionine (13  $\text{mmol}/\text{dm}^3$ ) and the catalyst. Riboflavin was added last and the reaction was initiated by illuminating the tubes with two 15 W fluorescent lamps. Equilibrium could be reached in 10 minutes. EDTA removes the disturbing trace metal ions, since the metal ion–EDTA complexes have no SOD activity. From the resulting graph the volume of enzyme mimicking complex corresponding to 50% inhibition ( $\text{IC}_{50}$ ) was registered to allow a comparison with the efficiency of the real enzymes and other SOD mimics. The enzyme mimics works the better when the  $\text{IC}_{50}$  is the smaller. There was no reaction without illumination and the support did not display SOD activity either.

## **Results and Discussion**

### *General considerations*

Even visual inspection of the samples revealed the success of building the complex on the surface of the support, since all the materials were colored – they were all blue.

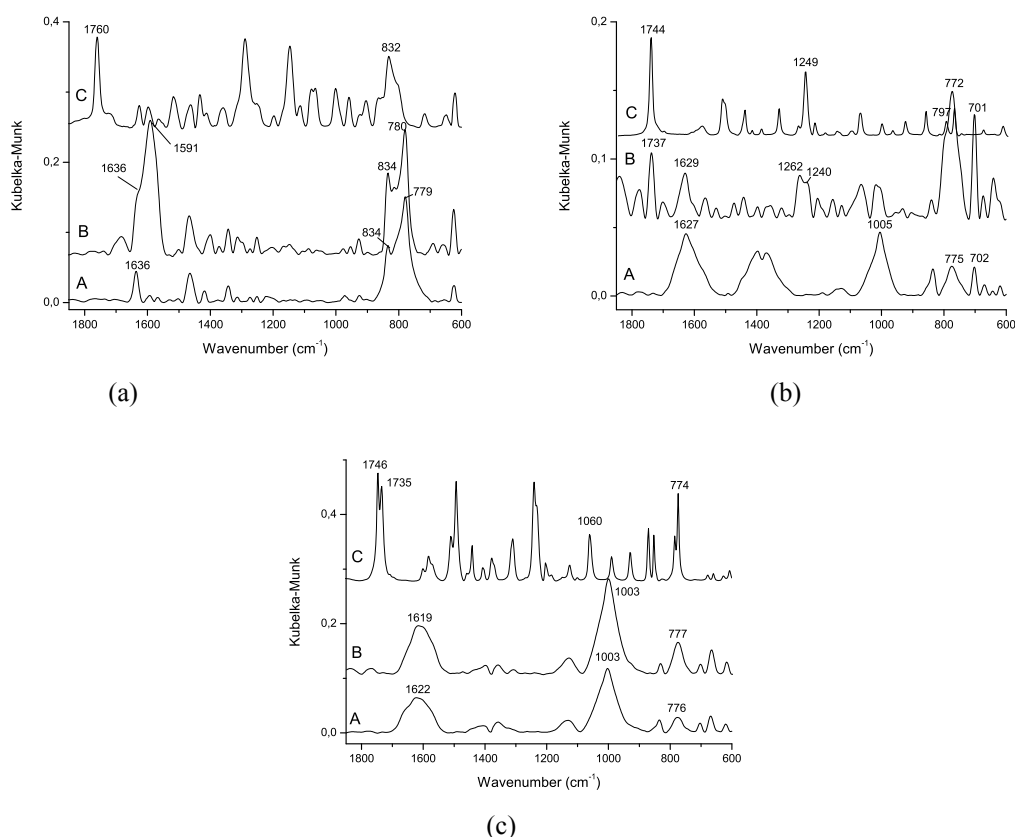
The blue color even indicated that the coordination of the Co(II) ion in the surface-grafted complexes was four.

As far as coordination modes are concerned it is known that the imidazole nitrogen is always a coordinating site [4], just as the sulfur atom(s) in the thiolate or thiol group or in the disulfide bridge. When the amino group is free and it is not the anchoring site its nitrogen is often a coordinating atom.

Whether the carbonyl group of the acid or the Boc protecting group is coordinated can be learnt from the IR spectra as shown in the following paragraphs.

*The FT-IR spectra of the surface-grafted complexes with uniform ligands*

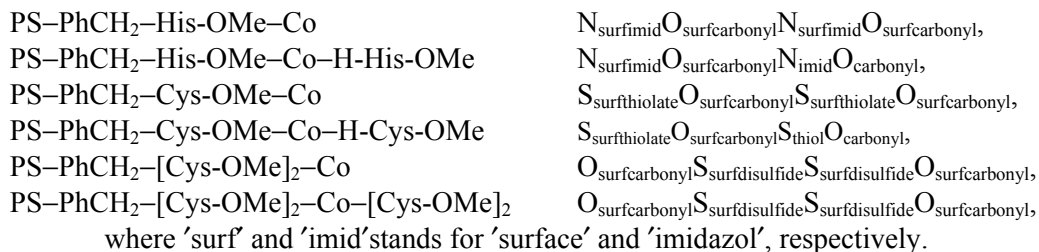
First, let us show some spectra of complexes having C-protected amino acids covalently anchored onto Merrifield's resin (Fig. 2).



**Fig. 2.** Co complexes with C-protected (a) histidine, (b) cysteine and (c) cystine, covalently anchored onto Merrifield's resin (traces A and B: the anchored complexes prepared under ligand-poor and ligand-excess conditions, respectively; trace C: the pristine C-protected amino acid). The spectrum of the resin is subtracted

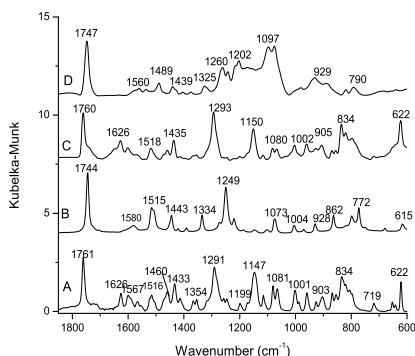
Analysis of the spectra reveals that under ligand-poor conditions (only surface-anchored amino acid molecules are available for complexation) the carbonyl stretching vibration(s) (near 1750  $\text{cm}^{-1}$  in the pristine C-protected amino acids) is (are) always shifted towards lower wavenumbers indicating that the carbonyl oxygen (or oxygens in

the C-protected cystine) is a coordinating site. It remains so for the added histidine methylester (Fig. 1/a) and the cystine dimethylester (Fig. 1/c). Nevertheless, while the spectrum changes for the former on the addition of excess ester, it is the same for the latter. This means that ligand excess does not result in the rearrangement of the surface complex in this latter case, while it does in the former one. However, the carbonyl oxygen of the added cysteine methylester (Fig. 1/b) does not take part in complexation, the position of its vibration is close to that of the pristine ester. These observations and the received wisdom summarized under the heading '*General considerations*' boil down to the following structural proposals:

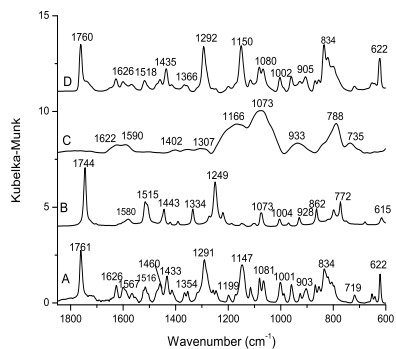


### The FT-IR spectra of the surface-grafted complexes with mixed ligands

Surface-anchored Co-C-protected amino acid complexes were built on the Merrifield's resin as well as the silica gel support. For the syntheses both the *A* and the *B* methods were used. Here, the FT-IR spectra corresponding to the silica gel-grafted Co-C-protected mixed amino acid complexes prepared by both methods are only shown (Figs. 3 and 4).



**Fig. 3.** Method A: the FT-IR spectra of *A* – H-His-OMe, *B* – H-Cys-OMe, *C* – SG-Cys-OMe-Co-His-OMe, *D* – SG-His-OMe-Co-Cys-OMe. The spectrum of SG is subtracted.



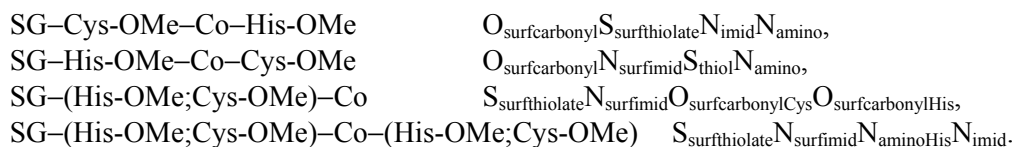
**Fig.4.** Method B: the FT-IR spectra of *A* – H-His-OMe, *B* – H-Cys-OMe, *C* – SG, 1:1 mixture, ligand-poor *D* – SG, 1:1 mixture, ligand-excess. The spectrum of SG is subtracted.

Traces *C* and *D* in Fig. 3 predominantly show the features of the added amino acid esters. Their carbonyl frequencies in the anchored complexes practically stay at the position of the pristine esters. In the anchored amino acids the carbonyl vibrations shift

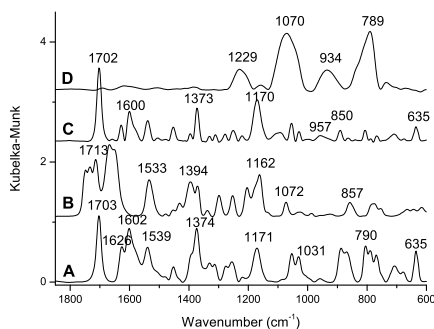
to about  $1630\text{ cm}^{-1}$  and to the  $1300\text{--}1000\text{ cm}^{-1}$  range in SG–Cys-OMe–Co–His-OMe and SG–His-OMe–Co–Cys-OMe, respectively.

Trace **C** in Fig. 4 shows a two-humped broad feature in the  $1300\text{--}1000\text{ cm}^{-1}$  region indicating that the carbonyl oxygens in both amino acid esters of the 1:1 mixture takes part in complexation, when only surface-anchored amino acid esters are available as ligands. Under ligand-excess conditions, however, the surface complex rearranges and histidine methylester will take the coordination sites not directly connected to the surface of the support.

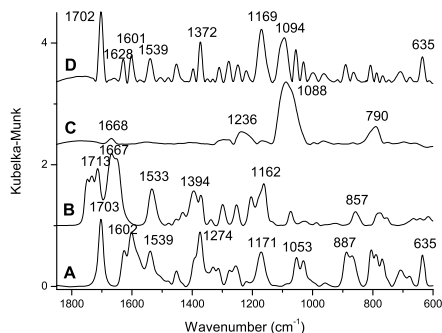
On the basis of these observations and the accumulated knowledge described at the beginning of the section, the following structures for the covalently immobilized complexes can be proposed:



Surface-anchored Co–N-protected amino acid complexes were built on the silica gel support only. For the syntheses both the *A* and the *B* methods were used. The FT-IR spectra of the materials obtained by both methods are displayed in Figs. 5 and 6.



**Fig. 5.** Method A: the FT-IR spectra of  
**A** – Boc-His-OH, **B** – Boc-Cys-OH,  
**C** – SG-O-Cys-Boc–Co–Boc-His-OH,  
**D** – SG-O-His-Boc–Co–Boc-Cys-OH.  
 The spectrum of SG is subtracted



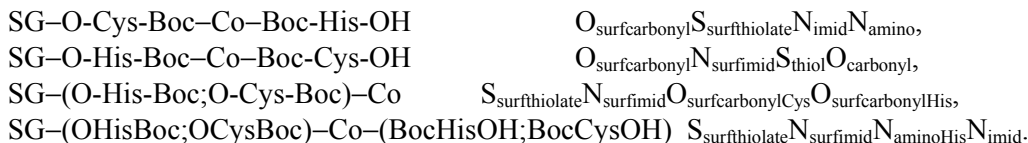
**Fig. 6.** Method B: the FT-IR spectra of  
**A** – Boc-His-OH, **B** – Boc-Cys-OH,  
**C** – SG, 1:1 mixture, ligand-poor  
**D** – SG, 1:1 mixture, ligand-excess.  
 The spectrum of SG is subtracted

Contrary to the C-protected analogs only trace **C** in Fig. 5 displays the features of the added amino acid, Boc-His-OH that is. Here, the carbonyl band remains where it was in the pristine amino acid, i.e., it does not participate in complexation. Trace **D** does not show any resemblance to any of the pristine amino acids. The carbonyl vibrations are shifted very much to the lower wavenumbers ( $1300\text{--}1000\text{ cm}^{-1}$  range) indicating that the carbonyl oxygens are coordinating sites in both amino acids.

When the 1:1 molar mixture was used for the synthesis of the surface-anchored complexes, under ligand-poor conditions a spectrum was obtained [trace **C** in Fig. 6 – SG–(O-His-Boc;O-Cys-Boc)–Co] showing striking similarity to spectrum **D** in Fig. 5 (SG–O-His-Boc–Co–Boc-Cys-OH). Here, the carbonyl groups of both amino acids are

sure coordination sites. Under ligands-excess conditions the surface complex rearranges and the Boc-His-OH from the added 1:1 mixture occupies the sites that are not directly involved in surface grafting.

On the basis of the above results and those described at the beginning of the section structural proposals for the surface-bound complexes are offered as follows:



### *Catalytic activity*

All materials displayed catalytic activity, i.e., could catalyze the dismutation of the superoxide radical anion. Catalytic activities differed widely though (Tables 1 and 2).

**Table 1.** *The SOD activities of the Co-C-protected amino acids covalently grafted onto Merrifield's resin*

Complexes	IC <sub>50</sub> (μM)
Cu-Zn SOD enzyme	0.4
PS-PhCH <sub>2</sub> -Cys-OMe-Co	10
PS-PhCH <sub>2</sub> -Cys-OMe-Co-H-Cys-OMe	184
PS-PhCH <sub>2</sub> -[Cys-OMe] <sub>2</sub> -Co	92
PS-PhCH <sub>2</sub> -His-OMe-Co-H-Cys-OMe	68
PS-PhCH <sub>2</sub> -Cys-OMe-Co-H-His-OMe	150
PS-PhCH <sub>2</sub> -(His-OMe;Cys-OMe)-Co	140
PS-PhCH <sub>2</sub> -(His-OMe;Cys-OMe)-Co-(H-His-OMe;H-Cys-OMe)	72

**Table 2.** *The SOD activities of the surface complexes covalently grafted onto modified silica gel.*

	IC <sub>50</sub> (μM)
SG-O-His-Boc-Co-Boc-Cys-OH	30
SG-O-Cys-Boc-Co-Boc-His-OH	18
SG-(O-His-Boc;O-Cys-Boc)-Co-(Boc-His-OH;Boc-Cys-OH)	28
SG-Cys-OMe-Co-H-His-OMe	80
SG-H-His-Co-H-Cys-OMe	50
SG-(Cys-OMe;His-OMe)-Co	35
SG-(Cys-OMe;His-OMe)-Co-(H-Cys-OMe;H-His-OMe)	47

Data reveal that there were catalysts coming close to the activity of the Cu-Zn SOD enzyme. This enzyme was used as comparison because of two reasons. First, Co-containing SOD enzymes do not exist, second, its catalytic activity has been measured and the result was available.

To much of our surprise, the most active anchored complex was the one having uniform ligands (PS-PhCH<sub>2</sub>-Cys-OMe-Co) supported on Merrifield's resin.

Previously, we thought that it would be one of the mixed ligand complexes constructed, since the surrounding of the cofactor in a metalloenzyme is usually non-uniform, various amino acid residues together with the metal ion(s) constitute the active site. We also thought that Merrifield's resin should be better support than silica gel, because its flexibility resembles the proteomic skeleton, the mobility of which contributes to the formation of the active site producing the best fit to the substrate(s) to be transformed. We were right in this respect, but only for the best catalyst.

The surface complexes with N-protected ligands also have appreciable activities, larger than those with the C-protected ones. In our view, the reason lies in the fact that the Boc protective group is much bulkier than the methoxy group, thus, these complexes accumulate more strain than those with the ester ligands and this excess strain makes them more reactive.

### Conclusions

It was possible to prepare Co–amino acid complexes grafted with covalent bonds onto modified silica gel or Merrifield's resin, both with uniform or mixed amino acids. The coordinating sites could be determined with the combination of literature data, FT-IR spectroscopy and chemical intuition. All the materials were catalytically active and some of them showed the promise of becoming efficient catalysts in electron transfer reactions in the laboratory or in fine chemical industry.

### References

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