Published in final edited form as:
Anal Chem. 2018 May 01; 90(9): 5909–5915. doi:10.1021/acs.analchem.8b00740.

Ascorbate oxidation by Cu(Amyloid-β) complexes: determination of the intrinsic rate as function of alterations in the peptide sequence reveals key residues for ROS production

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Abstract

Along with aggregation of the amyloid-β (Aβ) peptide and subsequent deposit of amyloid plaques, oxidative stress is an important feature in Alzheimer's disease. Cu bound to Aβ is able to produce Reactive Oxygen Species (ROS) by the successive reductions of molecular dioxygen and the ROS such produced contribute to oxidative stress. *In vitro*, ROS production parallels the ascorbate consumption, where ascorbate is the reductant that fuels the reactions. Because the affinity of Cu for Aβ is moderate compared to other biomolecules, the rate of ascorbate consumption is a combination of two contributions. The first one is due to peptide-unbound Cu and the second one to peptide-bound Cu complexes. In the present article, we aim at determining the amounts of the second contribution in the global ascorbate consumption process. It is defined as the intrinsic rate of ascorbate oxidation, which mathematically corresponds to the rate at an infinite peptide to Cu ratio, i.e. without any contribution from peptide-unbound Cu. We show that for the wild-type Cu(Aβ) complex, this value equals 10% of the value obtained for peptide-unbound Cu and that this value is strongly dependent on peptide alterations. By examination of the dependence of the intrinsic rate of ascorbate oxidation, followed by UV-Vis spectroscopy, for several altered peptides, we determine some of the key residues that influence ROS production.

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Notes
The authors declare no competing financial interest.

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Cu is an essential element for most organisms. As a redox active metal ion, it mainly occurs in the Cu(I) and Cu(II) states and is implicated in several key processes. It mostly acts as a catalytic centre in enzymes, like cytochrome c oxidase or superoxide dismutase, in which this metal cation is bound in a well-defined coordination site.1–6 Cu metabolism is tightly regulated and Cu is almost exclusively bound to proteins;7–9 when it is not the case, the resulting so-called loosely bound Cu is able to catalyse efficiently the production of reactive oxygen species (ROS). A well-documented case is the Cu overload in Wilson's disease, in which Cu is present in excess and, hence, part of the Cu pool remains loosely bound and thus prone to produce ROS.8,10 Another case where Cu can be detrimental are the complexes formed by Cu bound to intrinsically disordered proteins/peptides such as amyloid-β (Aβ), α-synuclein (αSyn), Prion proteins, etc.11,12 Aβ has been linked to Alzheimer's disease (AD)13–17 and it was proposed that Cu bound to Aβ is competent in ROS production and hence could contribute to the oxidative stress observed in AD.16

This currently leads to a strong research activity towards the better understanding of the structure and redox activity of the Cu complexes of Aβ in vitro.18–22 The complexes of Cu(I) and Cu(II) with monomeric Aβ are relatively well described, mainly by studies based on the use of the truncated soluble metal-binding domain $A\beta_{1-16}$16 (sequence DAEFRHDSGYEVHHQK). Aβ has no well-defined 3D structure in its monomeric state23 and this feature remains even after the coordination to Cu(I) or Cu(II), because the coordination is very dynamic and several different coordination spheres are in fast equilibrium.11 The most populated coordination sites at physiological pH for Cu(I) and Cu(II) to Aβ are presented in Scheme 1 and have been the subject of intense investigations in the last decade (for recent review, see 24). The main coordination sites are different between Cu(I) and Cu(II), implicating different ligands. More precisely, Aβ is mainly bound to the Cu(II) ion via the N-terminal amine, the adjacent Asp1-Ala2 carbonyl group, the imidazole groups from His6 and His13 or His14 and to Cu(I) via the two adjacent His13 and His14.16 This implies that a large reorganization energy is needed to pass from Cu(I) to Cu(II) and vice-versa,16 as probed by electrochemistry.18 Cyclic voltammetry studies show that direct electron transfer is extremely slow for Cu(I) and Cu(II) in their most populated states, noted here resting states.22 This electrochemical and further studies in homogeneous solution thus suggested that an "in-between" state is responsible for the redox reactions and ROS production (Scheme 1).18,22,25

Structurally, this "in-between" state (IBS) is different from the "resting states" (RS) but is in equilibrium with the RS of the Cu(I)-Aβ and Cu(II)-Aβ complexes. Functionally, the IBS is extremely fast in redox cycling because the environment of the metal centres is very close for the two redox states, requiring a minimal reorganization energy to switch from one redox state to the other one. The electrochemical18 and chemical redox reactions thus proceed via this IBS.22 It has been proposed that in the IBS, the Cu ion is surrounded by the N-terminal amine, the carboxylate group from Asp1 and one imidazole group from one His.18,22,25

In addition to the structure of the binding sites, another important parameter that has been addressed in vitro is the affinity of Aβ for Cu(I) and Cu(II). For Cu(II) the order of magnitude of the conditional dissociation constants at pH 7.4 (without any competitor like buffer) is mostly consensual, i.e. 10^{-10} M for $A\beta_{1-16}$ (Table 1). For Cu(I) the values are less

clear but they are in the order of 10^{-7} to 10^{-10} M.† For in vitro studies, it means that in a solution containing stoichiometric concentrations of Cu and Aβ, a fraction of Cu is not bound to the peptide due to the moderate affinity, which could be non-negligible at low working concentrations (μM range). Such peptide-unbound Cu species can seriously influence global ROS production because peptide-unbound Cu is extremely competent in ROS formation.

In the present work, we determine the intrinsic rate of ascorbate oxidation by Cu(Aβ) for a series Aβ peptides, the proposed Cu(II) and Cu(I) sites for the altered peptides being given in Figure S1. It corresponds to the rate of ascorbate consumption at super-stoichiometric Aβ to Cu ratio, i.e. where the contribution of peptide-unbound Cu is extrapolated to zero. As previously described,22,26,27 this value mirrors the ROS production rate. The comparison of the rates obtained for a series of Aβ peptides does confirm our recent proposition of the IBS structure.22 In addition, as a side result, we show that the peptide to Cu stoichiometry required to neglect the contribution of unbound copper in the overall ascorbate oxidation strongly depends on the peptide sequence.

Experimental Section

Chemicals

All the chemicals were purchased from Sigma-Aldrich (Germany). Ascorbate (Asc) solution 1 mM was freshly prepared before each set of experiments by dissolving L(+)-ascorbic acid sodium salt in Milli-Q water. The solution was kept on ice between measurements; no degradation affecting the ascorbate oxidation measurements was observed within that time. Cu(II) stock solution 0.1 M was prepared from $CuSO_4 \cdot 5H_2O$ in Milli-Q water. The concentration was verified by absorption spectroscopy at 800 nm (ε = 12 M^{-1} cm^{-1}). HEPES buffer solution 0.1 M, pH 7.4, was prepared from 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid and NaOH.

Peptides

$A\beta_{1-16}$ peptide (sequence DAEFRHDSGYEVHHQK) and the several mutants with purity grade >95% were purchased from GeneCust (Dudelange, Luxembourg. Stock solutions of about 2 mM of the peptides were prepared by dissolving the powder in Milli-Q water (resulting pH≈2). Peptide concentration was determined by UV-vis absorption of Tyr10 considered as free tyrosine (at pH 2, ε_{276}-ε_{296} = 1410 $M^{-1}cm^{-1}$).28 The $A\beta_{1-16}$ peptide is regarded as a pertinent model of the full-length peptide when studying metal related processes (except aggregation).28

αSyn_{1-6} peptide (sequence MDVFMK) was synthesized on solid phase using Fmoc chemistry as reported in ref. 29. Rink-amide resin was used as the solid support so that the resulting peptides will be amidated at the C-terminus. After removal of the peptides from the resin and deprotection, the crude products were purified by RP HPLC on a Phenomenex Jupiter 4u Proteo column (4 μm) 250 x 10 mm, using a Jasco PU-1580 instrument with

†The discrepancy originates from the Cu(I) affinity value of the dye used in the UV-Vis competition experiment.

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diode array detection (Jasco MD-1510), using a semi-linear gradient of 0.1% TFA in water to 0.1% TFA in CH_3CN over 40 min. The identity of the peptide was confirmed by electrospray ionization mass spectrometry (Thermo-Finnigan). The purified peptide was lyophilized and stored at 20 °C until use. Stock solutions of the peptide were prepared by dissolving the powder in Milli-Q water. Peptide concentration was determined by UV-vis absorption of Phe4 considered as free phenylalanine ($\varepsilon_{257.5}$-ε_{280} = 193 M^{-1} cm^{-1}).

Ascorbate oxidation

The ascorbate oxidation was monitored as a function of time by absorption spectroscopy at 265 nm in 50 mM HEPES buffer, pH 7.4, containing 100 μM of ascorbate and peptide concentrations ranging from 0 to 100 μM. Ascorbate oxidation, and therefore ROS production, was initiated by Cu addition. Order of additions: 1st ascorbate, 2nd peptide, 3rd Cu(II). This order of additions allows (i) checking that there is no ascorbate consumption by the peptide alone that could be due to small contamination with redox metal ions in the peptide batch and (ii) that the ascorbate concentration is correct (given by the absorbance value). Addition of ascorbate to trigger the reaction was also tested but it implies to start the measurement and analysis with an unknown ascorbate concentration since during the mixing of ascorbate, a significant amount can be consumed when the reaction is fast (id est when peptide to Cu ratio is weak or in absence of peptide). The initial ascorbate oxidation rate was calculated taking into account the slope of the variation in Asc concentration (obtained by dividing the variation in absorbance by the extinction coefficient of Asc, ε = 14500 $M^{-1}cm^{-1}$) for ca. 60 s. Measurements were performed at least three times at 25 °C, with various stock solutions of peptides and Cu. Experiments were performed with a diode-array spectrophotometer Agilent 8453 UV-Visible equipped with a Peltier temperature controller.

The initial ascorbate oxidation rate of free Cu at 10 μM varies from one experiment to another and has an average value of 0.051 μMs^{-1} (± 0.005). The average value has been determined by more than 30 experiments performed on different days. As can be seen from the standard deviation, the initial ascorbate oxidation rate of free Cu undergoes some (reasonable) variations from one series to another, even theoretically under the very same experimental conditions. Some parameters can explain such variations: the exact (real) Cu and Asc concentrations, pH, correct oxygenation of the solution have a dramatic influence on the rate measured. That is the reason why we have let this parameter adjustable in the reproduction of the theoretical curves. In addition, we have evaluated that the change in the initial ascorbate oxidation rate of free Cu has a significant impact neither on the K^P nor on the r_i^P values of the Cu(peptide) complexes, as determined below. The initial ascorbate oxidation rate (i. e. the rate obtained by taking the slope of absorbance decrease for ca. 1 minute after the addition of Cu(II)) was used to get rid of any possible well-documented peptide oxidations that would lead to modifications of the species under studies.30,31

Experiments were performed at [Cu] = 10 μM to be able to detect measurable change in the rate of ascorbate oxidation. In addition, such a value can be considered as the highest limit with respect to biological concentrations. This implies that the results obtained here can be extrapolated to biological concentrations, since up to 10 μM the r_i^P value is independent of

the Cu concentration (compare data obtained at 5 μM in Figure S4 and 10 μM in Figure 1, Table 1).

Results and Discussion

Determination of r_i^P, K^P and *thv*

Previous works from different groups showed that the ascorbate oxidation followed by absorption at 265 nm is an easy and robust method to evaluate the catalytic capacity of Cu-compounds to generate ROS.26,27,32–37 Indeed, ascorbate oxidation paralleled the production of H_2O_2 and HO• as measured by other methods according to the following reactions:

$$2\text{Asc} + O_2 + 2H^+ \rightarrow H_2O_2 + 2\text{Asc(ox)}$$

$$\text{Asc} + H_2O_2 \rightarrow HO^{\bullet} + HO^- + \text{Asc(ox)};$$

In order to evaluate the intrinsic ascorbate oxidation ability of the various Cu(Aβ) complexes, we measured the ascorbate oxidation rate at different peptide to Cu ratios for several Aβ peptides (Figure 1 and S2). Figure 1 shows the three curves obtained for $A\beta_{1\text{-}16}$, Ac-$A\beta_{1\text{-}16}$ and (H6A-H13A)$A\beta_{1\text{-}16}$ as a matter of illustration, other curves being given in the Supporting Info. The curves obtained differ on two features: (i) the asymptotic value obtained for super-stoichiometric ratios of peptide that corresponds to the intrinsic rate of ascorbate oxidation (when the system is freed from any contribution from peptide-unbound Cu), noted r_i^P, with the Cu complexes of (H6A-H13A)$A\beta_{1\text{-}16}$ and Ac-$A\beta_{1\text{-}16}$ being one of the most and the less active in ascorbate oxidation, respectively ; (ii) the curvature reflected by the parameter K^P, with Ac-$A\beta_{1\text{-}16}$ having the steepest one.

The curves obtained were fitted according to the following equations: $r_{obs} = -\frac{dA}{\varepsilon \ell dt} = r_i^f(1-\alpha) + \alpha\, r_i^P$ where r_{obs} is the rate of ascorbate consumption measured in Ms^{-1} for any given P:Cu ratio, α is the proportion of Cu bound to the peptide (regardless of the oxidation state), r_i^f and r_i^P are the coefficients describing the ascorbate consumption of peptide-unbound and peptide-bound Cu, respectively. $r_i^f = r^f(1 + corr[Cu])$, where $corr$ is a correction coefficient that takes into account deviation from linear dependence of r_i^f with the Cu concentration (See Supporting Info. for details, Figure S2). K^P is defined as $K^P C_0 = \frac{\alpha}{(1-\alpha)(s-\alpha)}$ where s is the P to Cu stoichiometry and C_0 the initial concentration in Cu (here 10 μM). During the calculation three parameters are adjusted: r_i^f, r_i^P and K^P, the $corr$ parameter being kept constant. To better illustrate the influence of r_i^P and K^P, especially on the contribution of peptide-unbound Cu in the ascorbate oxidation, we plot several theoretical curves by varying the r_i^P and K^P values (Figure 2 and S3). Note that contribution from $Cu(A\beta)_n$ species at high peptide to Cu ratio was discarded based on previous study.38 (and refs. therein)

In these plots, we also indicate by vertical arrows the peptide to Cu ratio above which the contribution of peptide-unbound Cu can be neglected (we arbitrarily, and for a matter of illustration, choose a threshold at 10% of the total rate of ascorbate oxidation). It then appears that the threshold value (noted *thv*) depends on the two parameters r_i^P and K^P. The higher K^P or r_i^P are, the lower is *thv*, meaning that higher K^P or r_i^P correlates to a lower contribution of free Cu in the total ascorbate oxidation rate. All the intrinsic rates r_i^P determined for the Aβ peptides are gathered in Table 1 and in Figure 3 (black bars).

Several comments can be made: (i) the range of r_i^P values is large, with extreme values separated by almost one order of magnitude, (ii) two families of peptides can be distinguished: His-Ala mutants, for which the corresponding Cu complexes show higher rates and N-terminally modified peptides for which the corresponding Cu complexes show lower rates than the reference Cu(A$\beta_{1\text{-}16}$) species. In the former case, it is worth noting that the higher the number of mutated His residues the higher the rate; and that H13A and H14A mutations display a more prominent effect than H6A. If we consider the equilibria shown in scheme 2 that depicts ROS production according to the RS - IBS model, the values of r_i^P mirror: (i) the intrinsic activity of the redox- active Cu(I/II)-peptide IBS and (ii) its population, which is dependent on the two interconversion constants K^I and K^{II}, corresponding to the equilibrium between the RS and the IBS for both +I and +II redox states, respectively.

The reactions in the case where there is no contribution from peptide-unbound Cu are shown in the dotted-line rectangle.

It is worth noting that changes in the r_i^P values as function of peptide alterations perfectly agree with the structure of the IBS we recently proposed (Scheme 1) and do not fit with the resting state. Indeed, higher r_i^P are obtained when two out of the three His are removed and lower r_i^P when the N-terminal part is changed, thus pointing the need of only one His in the IBS and the key role played by Asp1, respectively. Since only one His is needed, the equilibrium towards the IBS is favoured for the double His mutants thus leading to an increase in r_i^P value on one hand; and on the other hand, as NH_2 and COO^- from Asp1 are key ligands, preclusion of their binding ability induces either a lower rate or a lower population of the IBS, leading to a lower r_i^P value. In addition, when His6 is the sole His conserved, r_i^P is the highest value indicating that among the three possible His, His6 might be the one leading to a more active IBS. It is also worth noting that the r_i^P value obtained for the Aβ peptide in presence of Zn is slightly higher than without Zn, in line with the use of one or two His for Zn binding in the heteronuclear Cu,Zn(Aβ) peptide.26,47

The K^P values are plotted in Figure 3 (grey bars). First, there is no obvious correlation between the r_i^P and the K^P values. Second, there is no relation between the K^P values and the affinity value of Cu(I) or Cu(II) in the RS of the peptide of interest (Table 1). For instance,

the double His13AHis14A mutant has a weaker Cu(I) affinity by more than two orders of magnitude,27 and the Ac-Aβ_{1-16} has a weaker Cu(II) affinity by more than two orders of magnitude compared to the reference Cu(Aβ_{1-16}) species,41 whereas for the K^P values, they show higher values. Finally, there is no clear trend between the peptide alterations and the K^P values. This strongly suggests that the K^P parameter is made of contributions from several equilibria (Scheme 2), at least between peptide-unbound Cu(I)/Cu(II) and peptide-bound Cu(I)/Cu(II) in the resting and IBS states, K^I and K^{II} ascorbate oxidation rate of the Cu complex when in the IBS. However, K^P is a key parameter because it documents the contribution of unbound Cu in the overall ascorbate oxidation rate (Figure 2). For those peptides with similar r_i^P values, higher K^P corresponds to a lower contribution of peptide-unbound Cu in the ascorbate oxidation (compare for instance Aβ_{1-16} and H6A-Aβ_{1-16}). The *thv* values, mirroring the contribution of peptide-unbound Cu in the ascorbate oxidation, are also plotted in Figure 2. They strongly depend on the peptide sequence, with no obvious trends.

Results obtained here strongly support the mechanism we previously proposed,22 by which ROS are produced by Cu(Aβ) complexes via an intermediate very-reactive state (IBS) in equilibrium with inactive resting states. The intrinsic rate of ascorbate for a large series of modified peptides were determined to get rid of any possible contribution from peptide-unbound Cu that would have biased the analysis of the data. From the results obtained here, we conclude that: (i) The three single His mutations have different effect, with H13A and H14A leading to higher rates than H6A, as previously pointed out,27 thus indicating that His6 may play a special role in the IBS; (ii) the same conclusion arises from data of the double His mutants, since the H13AH14A mutant shows the highest rate; (iii) modifications of the N-terminal part either by amidation of the side-chain or acetylation of the amine induces a decrease in the rate, indicating that both of the Asp1 binding groups are present in the IBS. It has recently been proposed that ROS production is governed by the respective amount of component I and component II, where component I is the most populated for the Cu^{II}(Aβ_{1-16}) at physiological pH, while component II is present as a minor form, where Cu(II) is bound to the N-terminal amine, the adjacent amide group, a carbonyl group from the peptide backbone and the sidechain of one His residue.16 Using modified peptides, the authors obtain Cu(II) complexes with either component I (A2P) or component II (D1A, D1E) environment.42 Complexes present in component II-only produce less ROS than the wild-type, in line with our results. Both models (*component I versus component II* or the *IBS*) can be reconciled if one considers that peptide modifications leading to component II-only complexes are the same that changes on the Asp1 binding ability. However, the *component I versus component II* theory disagrees with the results we obtained here for the double His mutants, which lead to component II species only at pH 7.422 but do produce (and by far) more ROS than the wild-type. In other words, *the component I versus component II* model may give an indication of the value of the K^{II} parameter (See Scheme 2) because in component II the RS of the Cu(II) species is more difficult to reduce. However, it does not document the overall process that depend on other (likely more important) parameters. In particular, in presence of such ascorbate concentration, the Cu ion is mainly in the Cu(I) state48 and thus K^I may contribute more than K^{II}.

Conclusion

In the present work, we were able to determine the intrinsic rate of ascorbate oxidation for Cu complexes of $A\beta_{1\text{-}16}$ and several altered Aβ sequences. The studies were performed so that the contribution of highly active peptide-unbound Cu could be neglected. It was demonstrated that care has to be taken when comparing reactivity of this type of peptides as the amount of contribution from peptide-unbound and peptide-bound Cu during the ascorbate oxidation at a given Cu to peptide ratio can significantly depend on the peptide sequence and cannot be predicted from the affinities of Cu(II) or Cu(I) in the RS. We were also able to show that, in contrast to what we previously proposed,26 Zn does have an impact, although weak, on the ability of Cu(Aβ) to produce ROS, since a higher intrinsic rate was observed in presence of Zn. In this context, it may be interesting to study familial mutants of Aβ in the future to identify whether some of them would have high ascorbate consumption rates that could be linked to their increased toxicity. Based on what we observed here for the H6A mutant, it can for instance be anticipated that the H6R English mutation will increase the rate of ROS formation.

Importantly, we have strongly reinforced the model of IBS we have recently proposed22 and we even refined it since we were able to show that His6 has a preponderant role (compared to His13 or His14).

In addition, the method described here has also been applied to another IDP, namely the αSyn (alpha-synuclein modelled by the first six amino-acids involved in PD,49 to illustrate that it could be widened to any IDP (Figure S2, table 1). Altogether, the data obtained here show that the highly dynamic character of Cu complexes with IDP,11 having different coordination spheres for Cu(I) and Cu(II), leads to a very intricate system regarding the ROS formation. Therefore, the main resting states are not enough to explain their chemical reactivity.

Finally, the value of intrinsic rate of ascorbate oxidation, r_i^P, by Cu(Aβ) complex represents 10% of that by peptide-unbound Cu. There are two main ways extrapolating this value to more biological conditions: (i) compared to ROS production by unbound Cu, the contribution of Cu(Aβ) is weak; (ii) but *in vivo*, Cu may not be “free” but linked to another biomolecule fully incompetent in ROS production and then the contribution of Cu(Aβ) is far from being negligible. In addition, apart from the intrinsic rate of ascorbate oxidation, other key parameters will influence the ROS production *in vivo*. They are the concentration in Cu and the Cu to peptide ratio that correspond to the K^P and *thv* described here.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge the support from the French National Agency of Research (ANR-13-BSV5-0016), and from the University of Strasbourg Institute for Advanced Study (USIAS). The authors also acknowledge Simone Dell’Acqua (Chemistry Department of the Pavia University) for the provision of the $\alpha Syn_{1\text{-}6}$ peptide.

Abbreviations

Aβ	Amyloid-β
Abs	absorbance
AD	Alzheimer's disease
Asc	ascorbate
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
IBS	in-between state
IDP	Intrinsically disordered proteins/peptides
PD	Parkinson's disease
ROS	Reactive Oxygen Species
RS	resting states
αSyn	α-Synuclein
wt	wild type

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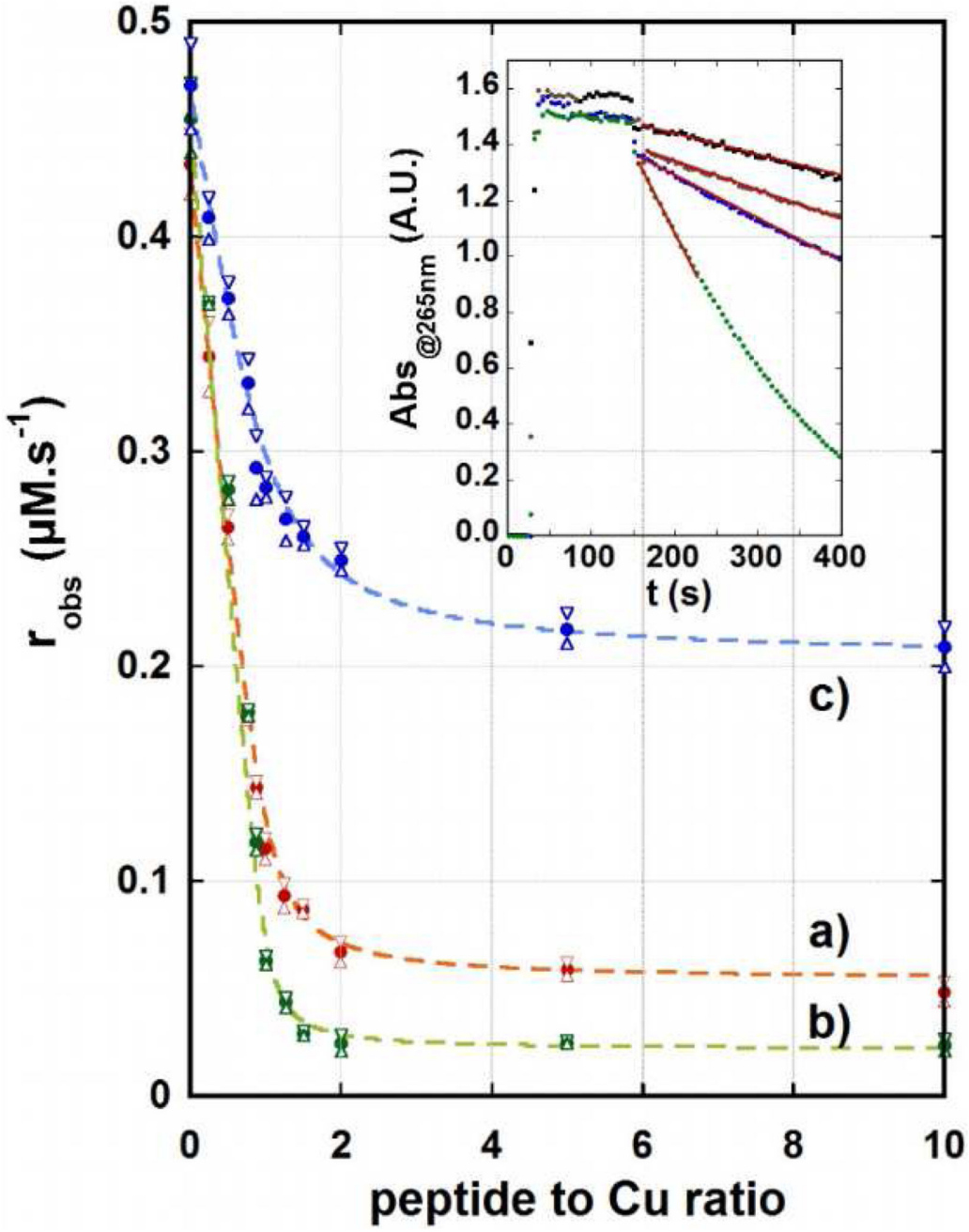

Figure 1.

Initial molar ascorbate oxidation rate as a function of peptide to Cu ratio: (a) red, $A\beta_{1\text{-}16}$, (b) green, Ac-$A\beta_{1\text{-}16}$ and (c) blue, (H6A-H13A)$A\beta_{1\text{-}16}$). Dots correspond to experimental data (given as mean ± S.D., n = 3), best fits are given as dashed lines. HEPES 0.05 M, pH 7.4; [Cu] = 10 μM, [Asc] = 100 μM. Inset: illustration of how the rate is experimentally determined.

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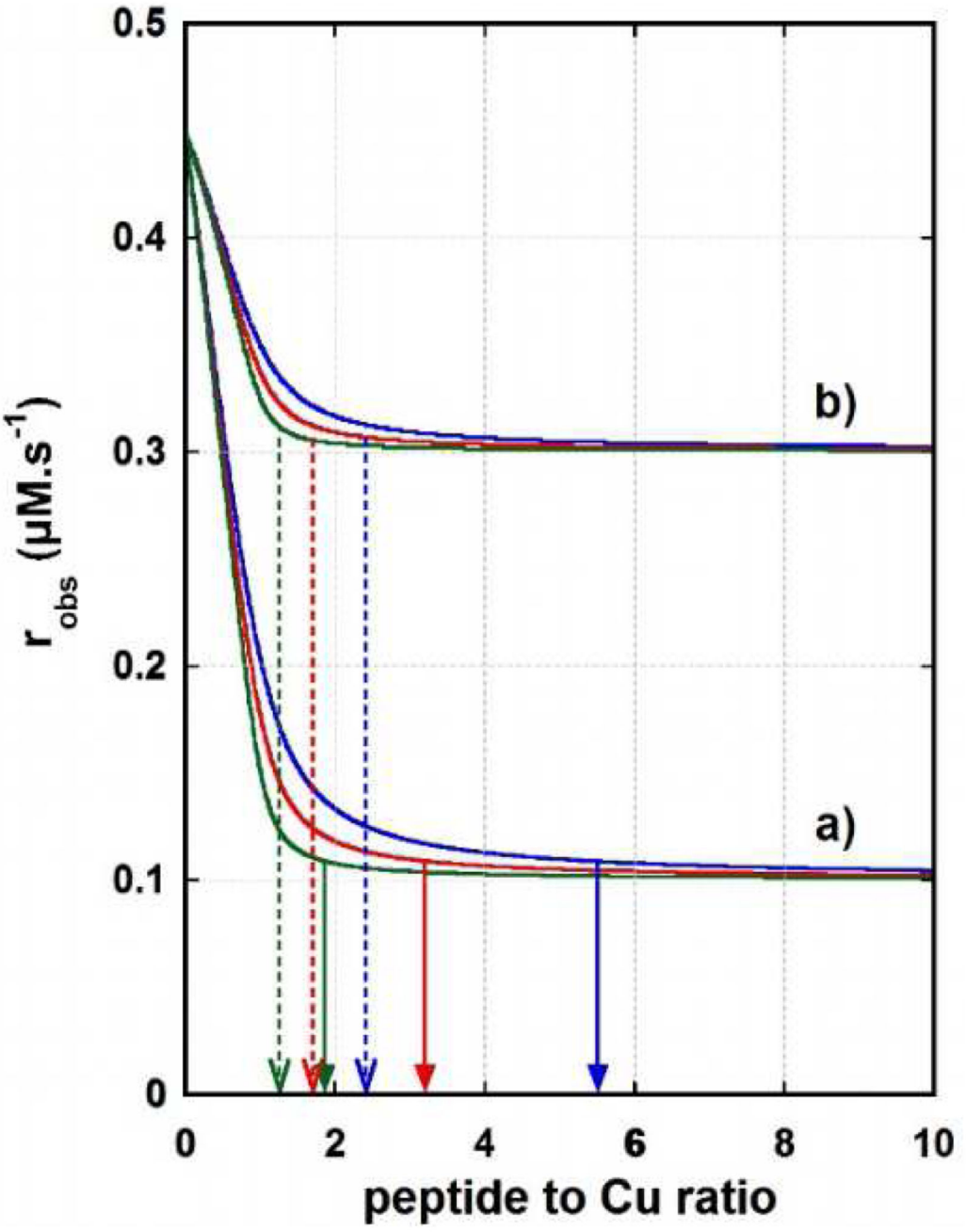

Figure 2.

Theoretical curves of r_{obs} as a function of the peptide to Cu ratio showing the influence of K^P and r_i^P values. a) r_i^P = 0.1 μMs^{-1} and b) r_i^P = 0.3 μMs^{-1}. Green lines: K^P = 5 x 10^6 M^{-1}; red lines: K^P = 2 x 10^6 M^{-1} and blue lines: K^P = 1 x 10^6 M^{-1}. Arrows indicate the values of *thv.* r^f = 0.52 μMs^{-1}, *corr* = 14 x 10^3 M^{-1}, [Cu] = 10 μM, [Asc] = 100 μM.

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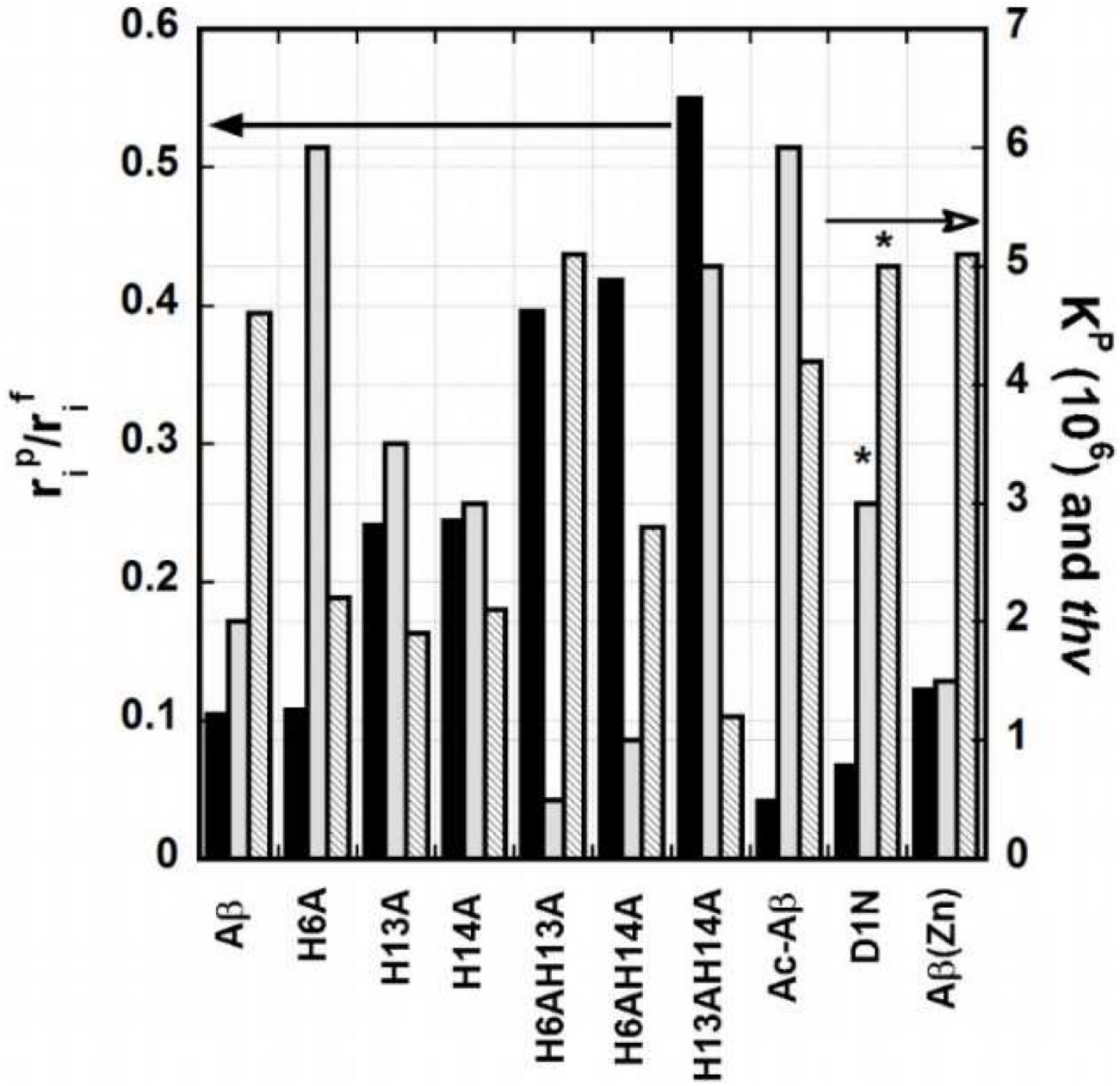

Figure 3.

r_i^P/r_i^f (black bars, left axis), K^P (grey bars, right axis) and *thv* (dashed lines, right axis) values for the series of Aβ complexes under study. r_i^f value equals = 0.051 μMs^{-1} (correspond to the average of all the available data). * = roughly estimated values (see Supporting Information for details).

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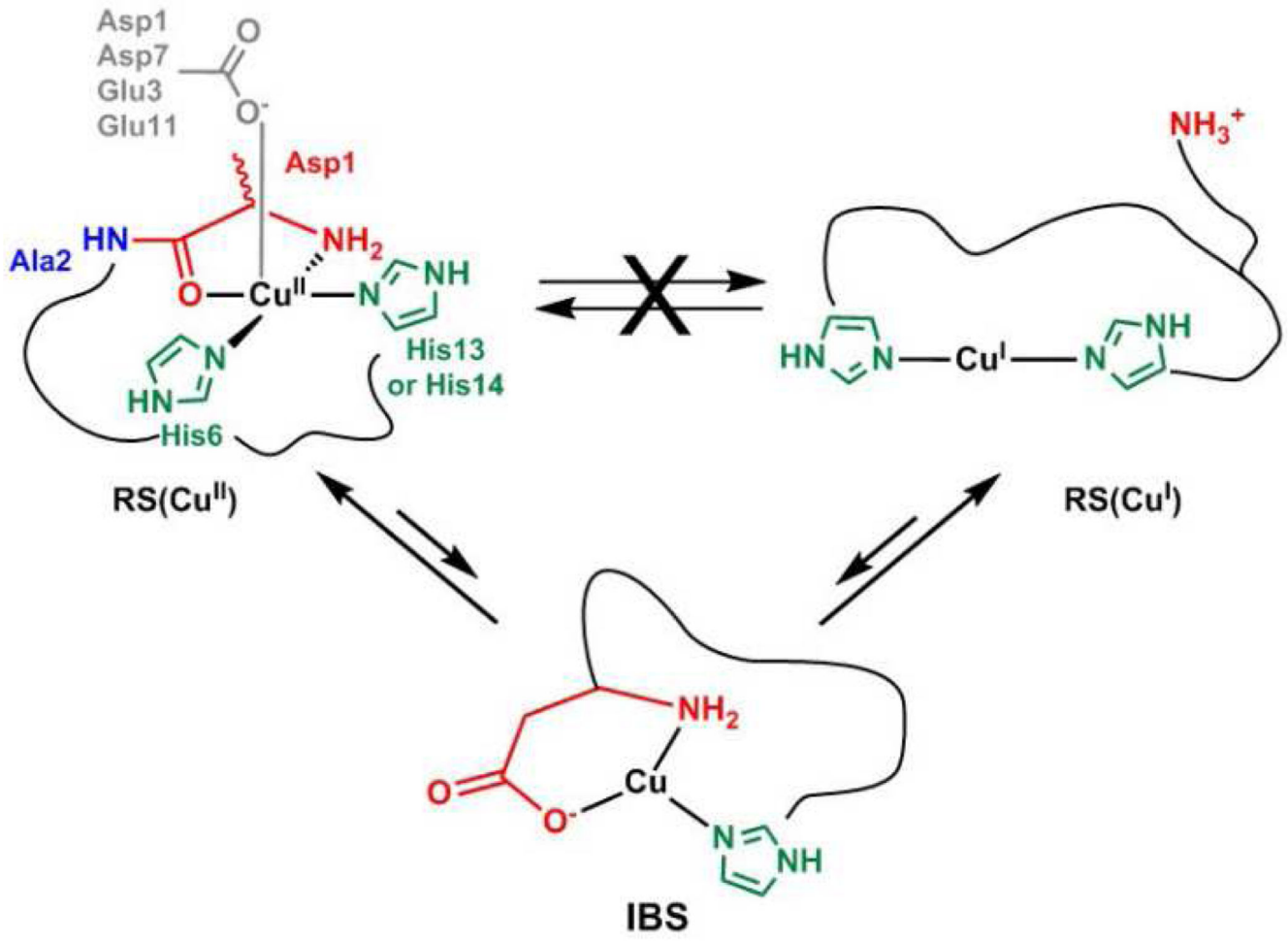
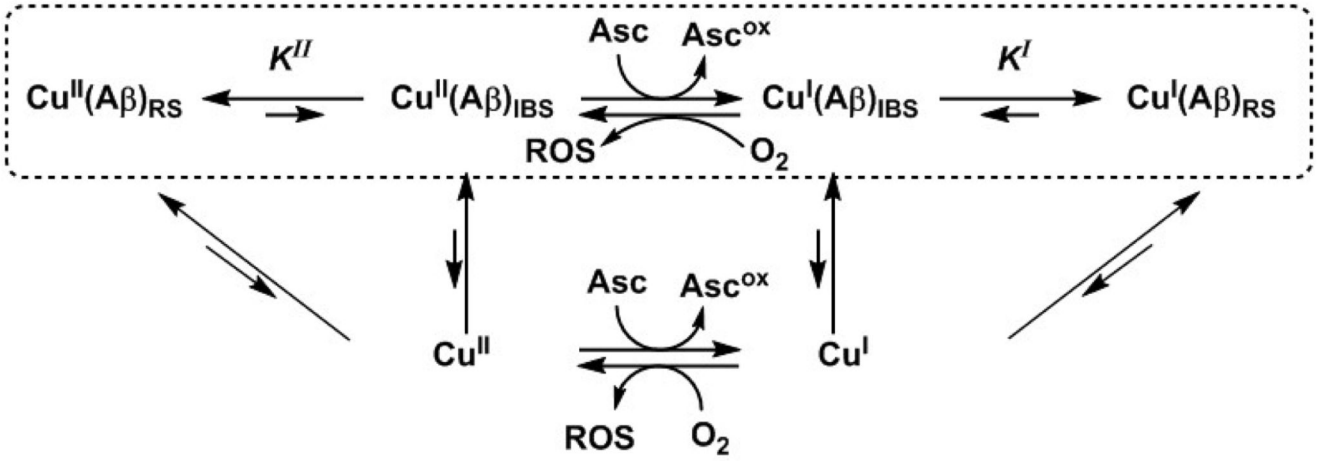


Scheme 1. Schematic view of the equilibrium between the RS and IBS of the Cu-Aβ complex during ROS production.



Scheme 2. Model of the reactions influencing the ascorbate oxidation rate of Cu-Aβ (and its modified counterparts).

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Table 1

Affinity values of Cu(I) $\left(K_a^I\right)$ and Cu(II) $\left(K_a^{II}\right)$ to the peptides, r_i^P, K^P and *thv* determined in the present work.

peptide	K_a^I (M^{-1} or *ratio*†)	Refs.	K_a^{II} (M^{-1} or *ratio*†)	Refs.	r_i^P (nMs^{-1})	K^P (10^6 M^{-1})	*thv*§
$A\beta_{1\text{-}16}$	7.5 x 10^6, 2.5 x 10^{10} ‡	27,39	10^{10}	27,40,41	54	2	4.6
H6A-$A\beta_{1\text{-}16}$	*0.4*	27,39	*0.32*	27	55	6	2.2
H13A-$A\beta_{1\text{-}16}$	*0.2*	27,39	*0.5*	27	124	3.5	1.9
H14A-$A\beta_{1\text{-}16}$	*0.3*	27,39	*0.5*	27	126	3	2.1
H6AH13A-$A\beta_{1\text{-}16}$	*< 0.004*	27	*0.1*	27	214	0.5	5.1
H6AH14A-$A\beta_{1\text{-}16}$	-		-		219	1	2.8
H13AH14A-$A\beta_{1\text{-}16}$	*< 0.02*	27	0.32	27	281	5	1.2
Ac-$A\beta_{1\text{-}16}$	*1.6*	39,42	0.02	27	21	6	4.2
D1N-$A\beta_{1\text{-}16}$	*1.6*	42	0.5	42	30	<5	
$A\beta_{1\text{-}16}$(Zn)	-			-	63	1.5	5.1
αSyn	10^5 - 10^6	43,44	10^7	45,46	8	1	>10

† K_a are given in M^{-1} for $A\beta_{1\text{-}16}$ and αSyn wild types, and as a *ratio* over the K_a of $A\beta_{1\text{-}16}$wt for the $A\beta_{1\text{-}16}$ mutants.

‡ The affinity constant values for Cu(I) differ from references 27 and 34, due to the different dyes used, which is then reflected for the mutant $A\beta_{1\text{-}16}$ peptides.

§ *thv* expressed as peptide:Cu ratio.