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# Recent Advances in Understanding the Electron Transport Through Metal-Azurin-Metal Junctions

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Azurin proteins are the workhorse of protein electronics. This is a branch of biomolecular electronics, a recent research field which investigates electronics based on biomolecules such as proteins, peptides, amino acids, bacterial nanowires or DNA. In general, the possibility of including biosystems in solid-state junctions has opened the way to the development of novel electrical devices, and proteins have attracted enormous attention thanks to their many interesting properties. In the particular case of metal-azurin-metal junctions, experimental measurements have revealed extremely efficient electron transport over large distances, showing conductance values which are higher than certain conjugated molecules of similar lengths. Moreover, the electrical current has often been found to be temperature-independent, which has been used as an evidence of coherent transport or quantum tunneling. Interesting effects have been observed, moreover, upon insertion of single amino-acid mutations. In spite of a huge amount of work, the exact mechanism for the charge flow through these systems is still under debate. In this review, we will revise the recent advances made in the electron-transport measurements of azurin-based junctions as well as the corresponding theoretical modelling. We will discuss the interpretation of the currently-available experimental results as well as the open issues which still remain to be clarified.

**Keywords: protein, azurin, biomolecular electronics, DFT, electron transport, molecular dynamics, amino acids, peptides**

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## 1 INTRODUCTION

The blue-copper azurin has been one of the workhorses for the field of protein-based electronics, which is one of the most recent branches developed out of molecular electronics [1–3]. Research on this subject aims at either exploring the use of biosystems of various kinds (such as proteins, amino acids, peptides, DNA and bacterial nanowires) in electrical devices or at mimicking their conductivity mechanism in bio-inspired electrical components [4]. Amongst the plethora of biosystems available in nature, proteins have attracted remarkable interest because of a large variety of properties which make them suitable for being incorporated into a multifunctional devices. These includes their capability of catalyzing a large number of reactions; their properties in terms of chemical recognition and selectivity; their mechanical and optoelectronic properties; their biocompatibility. The use of proteins has indeed been proposed for sensors, electrical noses, solar cells, field-effect transistors and flexible implants [5–7]. One of the traits which make proteins promising candidates for active components in electrical devices is their extremely efficient electron-

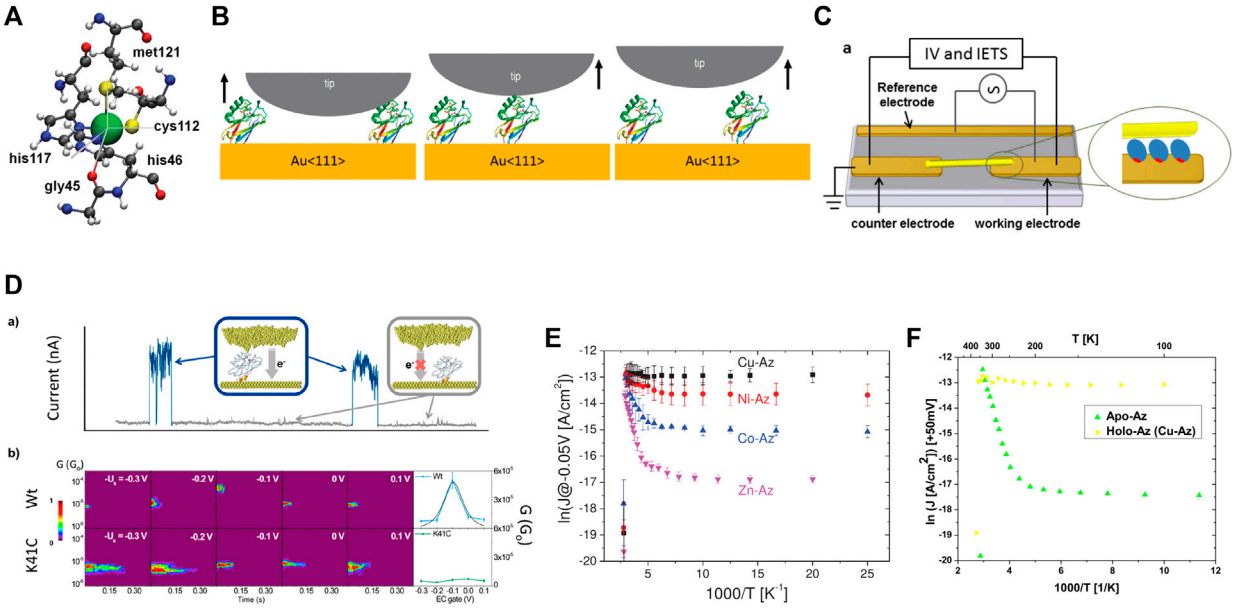


**FIGURE 1 | (A)** First coordination sphere of the Cu complex [56]; **(B)** Schemes of a single-azurin junction formation by the STM-BJ approach [24]; **(C)** Scheme of the Azurin junction fabricated by trapping nanowires by an ac electric field [26]; **(D)** Representative "blinks" (blue traces) identified in the transients of the current flowing between the two electrodes at a constant distance (upper panel) and Semilog 2D-blinking maps for the wild type blue-copper azurin (Wt) and K41C mutant at different EC gate potentials (lower panel) [38]; **(E)** ln(J) versus 1,000/*T* of Cu-Az, Ni-Az, Co-Az, and Zn-Az at –50 mV bias [42]; **(F)** Experimental ln(J) versus 1,000/*T* of Apo- and Holo-Az junction at +50 mV bias [41].

transport properties over large distances [3,8]. This is indeed the case for azurins, which are a type of redox metalloproteins involved in the denitrification processes, whose function is related to electron shuttling between cytochrome enzymes in the respiratory chain [9,10].

Extensive characterization on blue copper azurins has been carried out over the years by means of multiple techniques which include X-ray diffraction [11,12], electron paramagnetic resonance [13,14] or nuclear magnetic resonance [15,16]. The work by Solomon's group was very important to unveil the electronic structure of the Cu site in azurin and related blue copper proteins using a combination of spectroscopic techniques and quantum calculations [9,17–21]. In azurins the Cu center is coordinated by two histidine nitrogen atoms and a cysteine thiolate group in a trigonal planar structure (**Figure 1A**). The first coordination sphere is completed by two axial ligands: a methionine thioether group and a backbone carbonyl oxygen atom of a glycine residue. Overall, the Cu complex embedded in the azurin proteins displays a distorted pentacoordinated trigonal bipyramidal structure [12,14].

In the last decade, the electron transport through redox copper proteins has been analyzed extensively [3,22–24]. Interest in this particular type of proteins stems from the unique interplay between optical and vibronic properties with electron transfer in these systems as well as their biorecognition capabilities [8,25–29]. In the following sections, we will review what has been studied and understood about the electron transport through azurins incorporated in solid-state junctions (i.e., electrode-azurin-electrode juctions). We will analyze the most recent advances from both an experimental and theoretical perspective. In particular, we will focus on the efforts made by the community to understand the charge transport mechanism and to which extent azurin proteins can be considered promising candidates for molecular bioelectronic applications.

The review is organized as follow: in **section 2**, we will review the most recent experimental conductance measurements carried out on azurin proteins; in **section 3**, we will review attempts made in the field to interpret the experimental results by use of phenomenological models; in **section 4**, we will discuss studies carried out by large-scale (mainly ab-initio) calculations. Finally, in **section 5** we will draw some conclusions to highlight what the main controversies are and what still needs further investigation. Note that, within each section, results and conclusions from the literature will not be presented in chronological order, but rather sorted into paragraphs devoted to discussing specific issues.

## 2 ELECTRON-TRANSPORT MEASUREMENTS

Several electron-transport measurements have been carried out on the blue-copper azurin over the years. The presence of native cysteins makes it possible to bind this protein to gold electrodes in a well-defined orientation. It was even suggested that inserting them at different locations could actually allow studying this system along many orientations [23]. In Ref. [24], STM (scanning

tunneling microscopy) break-junction measurements on single-azurin junctions between a Au substrate electrode and an electrochemical scanning tunneling microscopy (STM) probe under bipotentiostatic control were reported. In this configuration, the tip is brought into contact with the protein-support gold surface followed by a fast pulling of the protein junction (**Figure 1B**). The measurements showed a transistor-like behaviour. The maximum conductance value was found to be $(2.1 \pm 0.7) \times 10^{-5} G_0$, with a 20-fold on/off ratio in the conductance between zero and high-electrochemical gate voltages. The results could be fitted to a two-step electron transfer model. The formation of a single-azurin junctions in a very similar experimental set up (in an electrochemical environment) was then also studied by keeping the STM tip at a fixed distance from the surface [8,24]. In this configuration, the contact between the STM tip and the protein is formed thanks to thermal fluctuations of the molecule which induce its spontaneous attachment or detachment to/from the tip. When the contact is established, sudden "blinks" are recorded in the current vs. time trace. These measurements showed evidence of switching events in spontaneously formed single-wire protein junctions. Moreover, the statistical analysis of the results seemed to confirm that the switching events at low bias voltages were related to the electrochemical gate potential of the system according to the redox behavior of azurin. Indeed, measurements on the Zn-azurin (a non-redox protein) within the same experimental set-up had showed no particular electrochemical-gate dependence [23,24]. Transition voltage spectroscopy was also proposed as a useful tool to study this system [30], showing that the transition voltage is strongly regulated by the electronic coupling of the protein with the STM probe. Results from STM imaging of azurin on gold can be found in Ref. [31].

The conductance *via* the blue copper azurin was also measured via conducting probe atomic force microscopy (CP-AFM), typically on self-assembled monolayers [31–35]. In this set up, the protein is trapped between an AFM conductive probe and a metallic substrate are used. The current flowing via application of a voltage bias is then recorded, while the feedback loop controls the pressure exercised by the probe. This makes it possible to measure topography, lateral forces, and current images simultaneously. An important output of these kinds of experiments were the observations on the temperature dependence of the conductance. In Ref. [32], at low forces the transport through azurin monolayer was found to be temperature independent, whereas at higher temperature ( > 310 K) and higher forces (6 nN) it was found to be thermally activated. Later on, in Ref. [34] the temperature independence of this protein was confirmed down to 4 K via experiments performed in liquid helium. In these experiments, the protein was connected to gold via a nanowire with a 200 nm diameter and bridging the gap between two photolithography-fabricated Au electrodes on $SiO_2$, leading to a contact area of the order of square micrometers. These results were claimed to support tunneling (rather than hopping) as the dominant transport mechanism in metal-azurin-metal junctions. For further discussion on the temperature dependence we refer the reader to **section 3**, where more experiments from the same research group will be discussed in relation with their theoretical interpretation.

An important detail which was found to affect the transport properties of this protein is the coupling to the electrodes. In Ref. [28,36], it was shown that modifying the coupling on one side of the junction can convert the transport mechanism from off-resonant to resonant without need of an external gate voltage. This was done within an experimental setup in which the top electrode was a gold nanowire which becomes connected to the protein upon landing on it (**Figure 1C**). A mercapto-propionic acid (MPA) group was bound covalently to the gold nanowire via an S atom and various types of chemical modifications allowed modification of the coupling. The importance of the contact at the protein-metal interface has been acknowledged also for other types of proteins [37]. The effects of inserting mutation was also explored in Ref. [38], where experiments revealed that inserting mutations in the blue-copper azurin can change the conductance mechanism drastically, changing the gate-dependent conductance curve from a Gaussian shape to a rather flat behaviour (**Figure 1D**). In Ref. [39] a 10-fold increase in switching of conductance was found for the N42C mutant, obtained by replacing the two native disulfide bridge-forming cysteine residues (3 and 26) by alanines, and the native asparagine residue 42 by a cysteine. This type of mutation affects the orientation of the protein within the junction and increases the coupling, causing higher current values. A complete discussion about the importance of having control over the protein-electrode interface can be found in a recent review reported in Ref. [40].

## 3 INTERPRETATION OF EXPERIMENTAL RESULTS BY PHENOMENOLOGICAL MODELS

The results reported above suggest that the exact nature of the electron transport through azurins is unknown and various possibilities should be taken into account. Indeed, over the years, many suggestions have been brought forward to try understanding the mechanism of transport. For instance, in Ref. [24,38], two-step tunneling models were considered, describing an incoherent type of process that involves partial relaxation in the Cu ion. There, the current was expressed in terms of reorganization energy, bias voltage, overpotential and model parameters describing the shift of the effective electrode potential at the redox center with the voltage and the overpotential.

Particular attention must be paid to a theoretical work by [41], in which various simple models were developed to try understanding the results of two experimental studies on self-assembled azurin monolayer carried out in different conditions. The first experimental study concerned measurements reported in Ref. [42], which employed Si-oxide substrate-Azurin-Au junctions or Hg lift-on-float-on (LOFO) heterojunctions. There, the voltage and temperature dependencies of Holo-Azurin (with Cu as the redox site) and Apo-Azurin (with Cu removed) had been compared, as well as the temperature

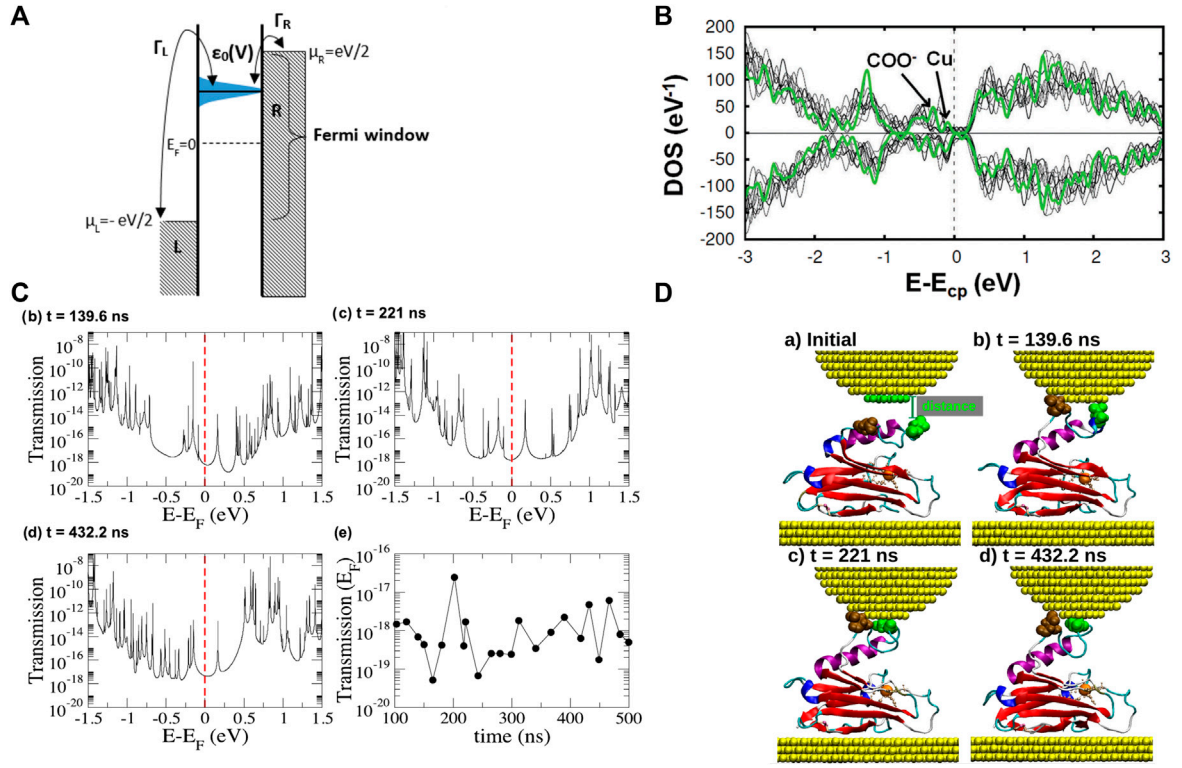


**FIGURE 2 | (A)** Schematic representation of the Landauer tunneling model [41]; **(B)** Spin resolved total density of the states of the azurin protein [56]; **(C)** Transmission as a function of energy for three selected Au-azurin-Au junctions obtained from molecular dynamics simulations and transmission at the Fermi energy for 23 representative geometries in the simulated blinking process [58]; **(D)** geometries corresponding to the transmission curves displayed in panel **(C)**.

dependencies of other metal-substituted azurins (Ni, Co., and Zn) (**Figure 1E**). The second experimental study was instead that reported in Ref. [26], which involved Au microelectrode-Azurin-Au micro-electrode heterojunctions and measurements of the current-voltage and current-temperature dependencies of holo-azurin. In both types of experiments, the current through the holo-azurin had been found to be approximately temperature-independent, but its bias voltage dependence of the holo-Az current in the two experiments differed considerably. In particular, an interesting observation in Ref. [42] concerned the apo-azurin which, in contrast with the temperature-independent holo-azurin, had shown activationless behavior at lower temperatures ($T < 200$ K) and activated behavior at higher temperatures ($T > 200$ K) (**Figure 1F**). For the other metal-substituted azurin monolayers, the temperature dependencies of the current interpolated between the Holo-Az behavior and the Apo-Az behavior. In the temperature-independent regime, the highest current had been found for the Holo-Azurin, while the lowest for the Apo-Azurin, with the other metal-substituted azurins showing intermediate current values between the two extremes (Cu-Az > Ni-Az > Co-Az > Zn-Az > Apo-Az) (**Figure 1D**). At the highest temperatures, the current magnitudes of all azurin types appeared to be similar. In order to shed light on the results of these two sets of experiments, in Ref. [41] the density current vs. voltage and vs. temperature were fitted to various models, namely the one-site hopping model, the Landauer off-resonant tunneling and resonant tunneling models, multisite extensions of these models, the extended fully adiabatic Newns-Anderson model, and the two-step ET model. Overall, this analysis suggested that Cu is likely to act either as a near-resonant/resonant tunneling or as an incoherent hopping site. It was suggested that, in the Holo-Az heterojunctions of Ref. [42], transport is mediated by incoherent resonant hopping through the Cu ion with also the possibility of coherent resonant tunneling through the same at low-bias voltages. In contrast, it was stated that, for the Holo-Az heterojunctions in Ref. [26], transport was mediated by off-resonant tunneling. For the Apo-Azurin experiments of Ref. [42] it was suggested that the removal of the Cu atom changes the transport mechanism to through-amino-acid off-resonant tunneling in the lower temperature (temperature-independent) regime and to through-amino-acid hopping in the higher temperature, activated regime. For the other metal-substituted azurins (Zn, Ni, Co.), it was found that the off-resonant tunneling model (based on a single bridge level) could reproduce the currents in the temperature-independent regime, and that the average tunneling barriers followed the same magnitude trend as the redox potentials of the metals. This seemed to indicate that the metal type plays a role in influencing the average tunneling barrier in these systems. For the high-temperature, activated

regime, the current behaviour could be reproduced by through-amino-acid hopping for all three metal substitutions, although it was found that the off-resonant tunneling model could also reproduce the activated region for Ni- and Co-substituted azurins. Very importantly, this study showed that a temperature-independent current does not necessarily imply a coherent tunneling mechanism, nor does it exclude a hopping mechanism. Nevertheless, this issue is currently still highly controversial [43]. In fact, coherent tunneling has been put forward in many studies concerning transport through azurins. In that case, a single-level tunneling model (**Figure 2A**) has been employed mostly to gain an insight into the experimental results [28,34].

## 4 LARGE-SCALE COMPUTATIONAL SIMULATIONS

The remarkable conductance measured in the experiments for the blue copper azurin is very surprising taking into account the low tunneling conductance which was computed for junctions based on single amino acids or single peptides [44–46], as well as its size (with a diameter of approximately 2.5 nm). This has inspired ab-initio-based calculations devoted to unraveling this puzzle. Until recently, most computational studies had either employed isolated partial fragments of the protein [47–51] or made use of quantum mechanics/molecular mechanics methods [52,53] which were not fully *ab-initio*. The study of the single Cu site or other fragment of the protein is, indeed, still currently fundamental for understanding many properties [54,55]. Nevertheless, an analysis of the electronic configuration of proteins when contacted to electrodes should not exclude any part of its physical structure. This is because the electron-transport properties through such hybrid structures are determined by a delicate interplay between the protein orbitals and the coupling to the leads, as it was mentioned in the previous sections. Unfortunately, studies of the whole protein structure had been hindered by computational limitations until very recently. Nevertheless, the current advances in computing performance have now made it possible to overcome this barrier. Recently, ab-initio (DFT) calculations have elucidated the electronic structure of the entire protein [56]. It was found that close to the Cu *d* levels are many orbitals localized on oxygen atoms which belong to carboxyl groups located all over the peripheral region of the protein (**Figure 2B**). This was later confirmed by an ab-initio study of the whole metal-azurin-metal junction [57]. In the same work, molecular dynamics simulations also reproduced the evolution of the typical junction created via the blinking technique described in **section 1** (**Figure 2D**). It was observed that the protein-metal contact is created upon separation of the $\alpha$ helix from the $\beta$ barrel. Interestingly, the DFT calculations showed that such a significant mechanical deformation does not affect the electronic structure of the Cu ion and of its first coordination sphere. Differences were, instead, observed in residues located either close to the tip or to the substrate or belonging to the intermediate region in which the $\alpha$-helix is separated from the $\beta$-barrel. Later, electron-transport calculations based on Green's function techniques, reproducing a coherent-transport mechanism, were carried out for geometries extracted from selected frames of these simulations [58]. The results there reported revealed that the tunneling conductance of those junctions would be extremely low (between $10^{-19}$ and $10^{-8}G_0$, see **Figure 2C**). This is due to the extreme low coupling of the Cu ion with the leads. Subsequent calculations [59] showed that similar results are obtained by replacing the Cu ion with other metals (Co., Ni and Zn). This can be ascribed to the fact that the main transmission background is provided by mostly glutamate and aspartate residues located on the common peripheral area, whereas the metallic ions give rise to very sharp resonances (originated, again, by their low coupling with the metal electrodes). Consequently, a striking similarity was observed between the conductance values calculated for the four proteins, in disagreement with the experimental results discussed in **section 3** [42]. Overall, these theoretical results cast doubts about whether the dominant role in the electron transport is played by the only metallic ion or whether, instead, other moieties provide a relevant contribution. This would be in line with the conclusions drawn in other recent experiments carried out on proteins [60] and protoporphyrins [61]. Furthermore, the low conductance values computed suggest that probably the transport mechanism involved in most of the experiments described in **section 2** and **section 3** is not fully coherent. It is worth mentioning that a low tunneling conductance was calculated by Green's function technique also for other kinds of proteins [62]. The results discussed above indicate that different theoretical models must be used to calculate the current flow in azurin-based junctions, probably by taking into account incoherent components.

The quest for a theoretical model which describes all the complex features of the transport through these kinds of systems is still actually underway for other types of proteins as well (see, for instance, the theoretical models applied to the study of the CTPR8 protein in Ref. [63] or Ref. myoglobin [64]). It is also worth mentioning that, in the context of incoherent transport through proteins, a crucial quantity is the reorganization energy. This quantity is the energy required to relax the structure of the protein and the surrounding solvent upon electron transfer. Thus it is equivalent to the energy difference between the product state in its equilibrium geometry and in the equilibrium geometry of the reactant state, or vice versa [65]. In the case of blue-copper proteins, the experimental value obtain for this energy is approximately 0.7 eV (see Ref. [66] and references therein), which is lower than in other metalloproteins. High reduction potential and low electron-transfer reorganization energy of azurin have been ascribed to a constrained inner-sphere copper geometry (see Ref. [67] and references therein). In Ref. [66], by means of a QM/MM study it was found that, in the case of azurin, the values of reorganization energy do not change when switching from dry to aqueous solution. Moreover, no significant reduction was

observed when contacting the protein to gold surfaces. These conclusions implied that a thermally-activate transport would be required.

In addition, the role played by mutations is currently still under investigation. Besides the effects on the protein-electrode coupling described in the previous sections, in Ref. [68] it was shown that single amino-acid mutations can induce quenching of protein vibrations, ultimately resulting in its overall stiffening. What the effect of this is on the electron-transport still needs to be clarified.

## 5 CONCLUSION

The nature of the exact mechanism of the electron transport through azurin-based junctions is still far from being understood. The two main controversies concern what the exact role played by the metal ion is and what kind of transport (coherent vs. incoherent) takes place in the system. While experimental studies from different groups have suggested either fully-coherent transport or 2-step tunneling mechanism, theoretical studies have already ruled out the possibility of fully-coherent transport and suggested the need of considering other types of transport. Hence, this puzzle still needs to be unraveled and further investigation is needed.

## AUTHOR CONTRIBUTIONS

CR-M and LZ wrote the manuscript, with input from all the authors.

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