Comparative Biological Chemistry of Cobalt Hemoglobin*

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SUMMARY

Cobaltohemoglobin A binds oxygen cooperatively with a Hill coefficient of up to 2.3 and its $p_{50}$ is found to be 91 mm. Its oxygen affinity is controlled by diphosphoglycerate which the protein binds very strongly. Cobaltohemoglobin A possesses only 40% of the alkaline Bohr effect of the native enzyme; carbon dioxide has no effect upon oxygen binding. The oxidation-reduction potential of cobaltohemoglobin A is +0.10 volt which is about 50 mv less positive than that of native hemoglobin. The electron paramagnetic resonance of the nitroxide spin label attached to the sulfhydryl groups of cys(92)$^*$ and the rates of reaction of these groups with paramercuribenzoate indicate that the “heme” crevice is more open near that residue. A detailed comparison of these properties with those of the native protein suggests subtle and intricate alterations of the quaternary structures of the oxy and deoxy species which result from substitution of the metal atom.

The initial successful preparation of cooperative $\cdot$OHb was reported that a large percentage of their reconstitutions were found to be difficult to reproduce. Thus, Hoffman et al. (3) with the finding that oxygen is $\pi$-bonded to cobalt in CoMb02. The discovery by Hoffman and Petering (1) that cobalt can be substituted for iron in bovine Hb$^+$ and sperm whale myoglobin with the retention of reversible oxygen binding property has opened an informative branch of hemoglobin chemistry. The electronic state of the cobalt nucleus in the modified protein can be examined with EPR (1). The single crystal EPR of CoMb and CoMbO2 was reported by Chien and Dickinson (2) with the finding that oxygen is $\pi$-bonded to cobalt in CoMbO2. In addition, comparison of the properties of the native and the cobalt proteins should help to elucidate the role of the metal atom in homotropic and heterotropic allosteric interactions.

The initial successful preparation of cooperative $\cdot$OHb was found to be difficult to reproduce. Thus, Hoffman et al. (3) reported that a large percentage of their reconstitutions were noncooperative, with $n = 1$, and this problem still remains. Furthermore, both $\cdot$OHb and $\cdot$OHbO2 are in low spin states (1, 2). If the cobalt protein truly possesses cooperative oxygen binding property, then some revision of the stereochemical theory is indicated. According to the current model stated in the most detailed form by Perutz (4), it is the transition from high spin iron in the deoxy form to low spin iron in the oxy form which triggers the homotropic interaction.

The central objective of this work is to prepare cooperative CoHbA from human hemoglobin, and to compare the properties of this protein with those of the native species in order to gain further insight into the role of the metal in hemoglobin. In this paper we present the results of oxygen equilibria (with and without DPG), carbon dioxide equilibria, alkaline Bohr effect, ultraviolet-visible spectra, oxidation-reduction potentials of CoHbA, spin label EPR studies, and stopped flow reactions with p-MB.

EXPERIMENTAL PROCEDURE

Preparation of Human CoHb—HbA was prepared from fresh human blood by the method of Perutz (5). CoHbA was prepared in 20 ml batches of approximately 1% concentration. This procedure has been described previously (2). Eighty percent of our preparations are cooperative ($n = 1.8$ to 2.3). Individual CoHbA batches did not seem to depend upon the apoglobin preparation, as both $n = 1$ and $n > 1$ could result from reconstitution of a given apoglobin preparation. All CoHbA batches were routinely analyzed for oxygen reversibility and Hill coefficient. The noncooperative ones were discarded.

CoHbA$^+$ was prepared from cobaltic protoporphyrin IX acetate by essentially the same procedure as above except that sodium dithionite is omitted. Fresh preparations of CoHbA$^+$ tend to leave a trail of free cobaltic porphyrin on a Sephadex column possibly indicating weakness of its binding to the globin. The CoHbA$^+$ preparations are free of CoHbA contamination as confirmed by the absence of EPR signals at $g = 2$ and 2.3.

Determination of Spectral Properties—The optical spectra of cobalt proteins were obtained with a Cary 14 spectrophotometer. The extinction coefficients in this paper are calculated on the basis of an extinction coefficient of 7.96 O.D./per cent for deoxy CoHbA at 5350 A as determined by lyophilizing several salt free preparations.

Measurement of Oxygen Equilibria—A 1-cm path length cylindrical cell, fitted with stopcocks and containing a small magnetic stirring bar, was used in these measurements. The protein concentration was typically 0.1% in pH 7.2 0.1 M phosphate buffer. Isobesticity at 5640, 5425, and 5270 A was monitored to detect protein denaturation. At 25°, each sample was used to determine three points on the oxygenation curve. Desired partial pressures of oxygen were achieved by first equilibrating...
the solution with an atmosphere of air or oxygen with constant flushing and stirring for 45 min. The cell was then pumped down to a pressure appropriate to the desired partial pressure of oxygen. Minimum total pressure above the solution was 200 mm and 30 to 40 min with gentle stirring was allowed for equilibration. Complete deoxygenation was accomplished by flushing the stirred protein solution with wet nitrogen gas for 45 to 60 min until no changes were observed in the visible spectrum.

DPG solutions were prepared from the pentacetylhexylammonium salt (Calbiochem, Los Angeles, California, Lot 000444) by treatment with Dowex 50W-X8 (6). Because of the relatively low stability of CoHb in salt free solutions, the DPG binding experiments were performed in 0.1 M NaCl. This salt concentration was selected as a balance between stabilization of the protein and suppression of the DPG effect on PI/Z. Some denaturation occurred as evidenced by the formation of small amounts of precipitate. As a consequence, there was some scatter in the Hill plot. The oxygenation curve was unchanged when our CoHb was taken through the procedure of Benesch et al. (6) which was devised specifically for the stripping of DPG. Therefore, our preparative procedure effectively removed DPG from the protein.

Determination of Alkaline Bohr Effect—The alkaline Bohr effect was determined by the method of differential titration (7). A thermally jacketed cell was fitted with a pH electrode (Radiometer Gk2321) and inlet and outlet for wet nitrogen or oxygen flush. Samples containing 0.33% protein and 1.0 M NaCl were deoxygenated by moderate stirring under nitrogen flush until the pH of the solution had stabilized for 10 min. The pH value was recorded and an oxygen atmosphere introduced. As soon as the drop in pH had stabilized, the solution was titrated back to the initial deoxy value with a 50-μl syringe filled with 0.01 N standardized NaOH.

Measurements were made on CoHbA as well as on native hemoglobin and also on iron reconstituted HbA. The latter was prepared by exactly the same procedure used to obtain cobalt reconstituted HbA.

Measurements of Oxidation-Reduction Potentials—Oxidation reduction potentials were measured potentiometrically in a thermally jacketed cell equipped with a cm² platinum electrode and a KCl-bridged saturated calomel electrode. A Honeywell 2730 potentiometer was used to monitor the equilibrium potential. The procedure was basically the mixture method of Antonini et al. (8).

Solutions of CoHbA and CoHbA⁺ (0.3%) in 0.1 M phosphate buffer were prepared as above. The protein concentration was determined by conversion to the same deoxy form with dithionite followed by spectrophotometry. Equimolar amounts of CoHbA and CoHbA⁺ were mixed under a constant N₂ flush with gentle stirring. Mediator (0.02 mole fraction of cobalt content) was introduced. Of the many mediators tried the ones which gave the most reproducible results and promoted rapid equilibration of potential are methylene blue for pH > 7.3 and thionin for pH < 7.3. Methylene blue can also be used at pH < 7.3. Control runs with native hemoglobin at pH 7.0 reproduced literatures values (9) to within 2 mv. Sodium phosphate buffer (0.1 M) was used below pH 8; 0.1 M sodium borate buffer was used above pH 8.

Spin Label Studies—Spin labeled CoHbA was prepared by reacting a 1% protein solution in 0.1 M pH 7.0 phosphate buffer with a 10-fold excess of iodoacetamide-nitroxide spin label (3-(2-iodoacetyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl, Lot 1201, Syva, Palo Alto, California). After 12 hours at 5°C, excess nitroxide was removed by gel permeation chromatography on a Sephadex G-25 (fine) column (20 cm x 3 cm²) equilibrated with 0.1 M pH 7 phosphate buffer. The EPR spectra of the spin labeled CoHbA were measured on a Varian E-9 spectrometer operating at 9.5 GHz.

Reaction of CoHbA with p-MB—p-Chloromercuribenzoate (Sigma Chemical Co.) was twice reprecipitated with acetic acid, centrifuged, and redissolved in 0.1 N NaOH. The stock solutions are 30 μM p-MB and 2.5 μM hemoglobin tetramer, both solutions being 0.1 M phosphate pH 7.0. The kinetics of PMB reaction with the sulfhydryl groups of hemoglobin was followed by monitoring the change in absorbance at 2540 Å which accompanies the formation of mercaptide. A Gibson-Durrum stopped flow apparatus was used for this work.

RESULTS

Optical Properties of Cobalt Hemoglobins—The optical spectra for CoHbA and CoHbA⁺ are given in Figs. 1 and 2. The spectra for FeHbA and FeHbA⁺ are also included for comparison. The Fe superscript is included in the abbreviations of the native species for the sake of clarity. The visible band of FeHbA is slightly lower in intensity and is characterized by a single broad peak at 5550 Å; the corresponding absorption of CoHbA is slightly lower in intensity and is characterized by a broad peak at 5500 Å. The absorption is not significantly altered by the oxidation state of the metal ion. The spectrum of CoHbA is shown in Fig. 1.

Fig. 1 (left). Optical spectra of FeHbA and CoHbA in pH 7.2 0.1 M phosphate buffer at 25°C. Ten-fold reduction of protein concentration was used to record spectra below 4500 Å. E values apply to spectrum above 4500 Å.

Fig. 2 (center). Optical spectra of FeHbA⁺ and CoHbA⁺ in pH 7.2 0.1 M phosphate buffer at 25°C. Ten-fold reduction of protein concentration was used to record spectra below 4500 Å. E values apply to spectrum above 4500 Å.

Fig. 3 (right). Optical spectrum of CoHbA⁺ in pH 7.2 0.1 M phosphate buffer at 25°C. Ten-fold reduction of protein concentration was used to record spectrum below 4500 Å. E values apply to spectrum above 4500 Å.
shoulder at about 5230 A. The latter feature was not resolved byloffman and Petering (1) for bovine "Hb, recent preparation of horse "Hb was reported to have a shoulder at 5150 A (10). The extinction coefficient at peak maximum for "HbA and Fe'HbA are virtually the same at 13 O.D./millimolar-heme. The \( \alpha \) and \( \beta \) bands of "HbA are found at 5660 and 5350 A as compared to 5770 and 5390 A in the case of Fe'HbA. The extinction coefficient for the \( \alpha \) band of "HbA is 0.8 of that of the visible band of Fe'HbA; the corresponding ratio is 1.2 for the native hemoglobin. The minimum between the \( \alpha \) and \( \beta \) bands of Fe'HbA is much deeper than that of "HbA.

The optical spectra of "HbA (Fig. 3) bears a close resemblance to the spectrum of Fe'HbA (Fig. 2). The 6300 A absorption of methemoglobin is totally absent in "HbA. Furthermore, the \( \alpha \) and \( \beta \) bands of "HbA and Fe'HbA are found at nearly identical wave lengths with almost the same extinction coefficients. In fact, if one were unaware of these facts, one could be misled into concluding that "Hb has a very strong affinity for oxygen when trying to deoxygenate a sample contaminated with the oxidized species.

The cochaloid protein has apparently little or no affinity for F\( ^{-} \) and N\( \tilde{\text{S}} \)\( ^{-} \) ions. Introduction of these ions at 0.1 M to "HbA caused no discernible change in its visible spectrum. However, it does have some affinity for the CN\( ^{-} \) ion. After introducing CN\( ^{-} \) to "HbA and after long standing, a stable spectrum results (Fig. 4) which is quite similar to the one for methemoglobin cyanide (11).

Cooperative Binding of Oxygen—A typical oxygenation curve for "HbA is shown in Fig. 5. The Hill coefficient is 2.3; the half-saturation partial pressure of oxygen is 91 mm. Also included in the figure is the curve for Fe'HbA (12). A Hill plot for "HbA is given in Fig. 6.

A physiologically important heterotropic linkage phenomenon of hemoglobin is the modification of oxygen affinity by organic phosphates. The effect of DPG on the oxygen affinity of "HbA was investigated. In order to perform this experiment, the "HbA protein which was prepared in phosphate buffer must first be thoroughly dialyzed to remove all the phosphate ions. It was found that the resulting salt free protein solution is rather unstable. There was rapid coagulation of "HbA at room temperature. Therefore, the measurements with DPG were made in 0.1 M NaCl. The pH of the solution was 7.3 before deoxygenation at 25°. As mentioned above our "HbA is stripped in preparation.

Like Fe'HbA (6), the affinity of "HbA for oxygen decreases with increasing DPG concentration (Table I). However, unlike the native protein, the effect for "HbA is linear with the DPG concentration and saturates at about 1 mole of DPG per mole of tetramer. In the case of Fe'HbA the effect is not saturated until the DPG concentration is about three times the hemoglobin concentration even in salt free solutions. The results in Table I show that each "HbA tetramer binds 1 mole of DPG as is the case for Fe'HbA, and that the "HbA-DPG complex is more stable than the Fe'HbA-DPG complex. Identical decrease of oxygen affinity by DPG has been reported for horse "Hb (10). The relative decrease for Fe'HbA, "HbA, and horse "Hb by saturation amounts of DPG, expressed as \( \Delta \log \alpha \), is 0.42, 0.48, and 0.48, respectively. Since the DPG effect on horse "Hb was not determined at less than saturation amount of DPG (10), it cannot be said whether the horse "Hb-DPG complex is also more stable than the native hemoglobin complex.

Alkaline Bohr Effect—The native hemoglobin releases 2.5 protons per tetramer upon oxygenation at physiological condition (7). It is this alkaline Bohr effect which promotes the transport of CO\( _2 \). "HbA is rather unstable in salt free medium and denaturation could invalidate the measurement. Additional protons could be released when other residues become exposed in the denatured form. Because of this concern, the Bohr effect was studied in 1.0 M NaCl. Of course high salt concentration is known to lower the absolute alkaline Bohr effect. Therefore, parallel measurements were made on native hemoglobin under identical conditions. Such a comparison would give the relative Bohr effect of "HbA referenced to the native hemoglobin.

![Fig. 4 (left). Optical spectrum of "HbCN in pH 7.2 0.1 M phosphate buffer and 0.1 M NaCN at 25°. Ten-fold reduction of protein concentration was used to record spectrum below 4500 A. ε values apply to spectrum above 4500 A.](image)

![Fig. 5 (center). Oxygenation curves for Fe'HbA and "HbA in pH 7.3 0.1 M phosphate buffer at room temperature. The data for Fe'HbA are taken from Reference 12, also reproduced in this work.](image)

![Fig. 6 (right). Hill plot of "HbA data of Fig. 5.](image)
The alkaline Bohr effect of native human hemoglobin A (○), iron reconstituted HbA (●), and CoHbA (■) as determined by differential titration (7). In each case the protein concentration was 0.33% in unbuffered 1.0 M NaCl.

Fig. 8 (center). Optical spectra of CoHbA in the presence and absence of CO2 in 0.1 M pH 7 phosphate buffer at 25°C. The curve designated CO2 was equilibrated to 722 mm of air plus 38 mm of CO2.

The oxidation-reduction potential for CoHbA as a function of pH. The same conditions employed by Antonini et al. (8), we have measured the oxidation-reduction potentials of CoHbA/CoHbA+ couple as a function of pH. The results are summarized in Fig. 9 along with those for the native enzyme (10). We also checked the latter values at several pH values. The potentials are referred to the normal hydrogen electrode.

The slopes of the E1/2 versus pH for the two proteins are identical at -0.06 volt per pH. To explain a similar dependence in cytochrome c, Theorell and Åkeson (16) suggested that the reduction is accompanied by dissociation of the reduced species. The proposed mechanism, adapted to hemoglobin (15), can be represented by

\[
\begin{align*}
F & = F_0 + \frac{RT}{nF} \left[ \ln \left( \frac{[\text{ox}]}{[\text{red}]} \right) + \ln \frac{1}{K} - pH \right] \\
E & = E_0 + \frac{RT}{nF} \left[ \ln \left( \frac{[\text{ox}]}{[\text{red}]} \right) + \ln \frac{1}{K} - pH \right]
\end{align*}
\]

where α is the degree of dissociation. Then the potential is given as

\[
E = E_0 + \frac{RT}{nF} \left[ \ln \left( \frac{[\text{ox}]}{[\text{red}]} \right) + \ln \frac{1}{K} - pH \right]
\]

where K, the equilibrium constant for Equation 1, is ≪[H+], and E is independent of pH when K >> [H+]. If this interpretation is correct, then both the iron and the cobalt proteins have the same heme-linked acid groups. They differ, however, somewhat in their pK values which are 6.5 for FeHbA and 6.9 for CoHbA. This small difference could have significant consequence, among others, in determining the conditions for crystallization of the two species (17).

Spin-labeled CoHbA—The EPR spectrum of iodoacetamide-nitroxide spin labeled CoHbA is shown in Fig. 10a. The spectral
intensity correponds approximately to two nitrooxide radicals per tetramer when compared to solutions of free spin label at known concentrations. The spectrum indicates a single type of binding site for the nitroxide. Oxygenation of the sample results in no discernible changes in the EPR spectrum. That is oxy and deoxy CoHbA have identical spin label EPR spectra. From the widths of the m_1 lines as compared to those of free nitroxide and no further spectral changes upon additional purifi-
cation of the spin-labeled CoHbA, the label can be definitely said to be bound to the protein.

The above observations are to be contrasted with the results of Ogawa (18) for native hemoglobin labeled with the same nitroxide. (Fig. 10, b and c). In this case, the spectra are much more anisotropic and there are dramatic differences between the oxy and deoxy species.

It is apparent from comparison of Fig. 10, a, b, and c that the attached spin labels differ in their mobilities in the two proteins. The EPR spectra of spin-labeled native enzyme showed that the label is intermediately immobilized; it is only weakly immobi-
lized in CoHbA.

Reactions of p-MB with CoHbA-To further investigate the protein conformation in the vicinity of the heme binding site, the rate of reaction of p-MB with the cys(92)p -SH groups was measured. The results are summarized in Table II. Our results for FeHbA O_2 are in excellent agreement with those of Geraci and Snda (19). There are two notable features about the results for FeHbA O_2. The first is that the mobility of the spin label becomes less restrained in oxyCoHbO_2 than it has when attached to FeHbA O_2. We take this to mean that the pH of unbuffered CoHbO_2 in 1.0 M NaCl is 6.82 compared to 7.33 for FeHbA under the same conditions.

There is a large reduction of alkaline Bohr effect when CoHbA is compared with native and iron reconstituted FeHbA under identical conditions. There is an accompanying decrease in CO_2 affinity with cobalt substitution. The implication is that there are fewer salt bridges in the deoxyferrohemoglobin than in the deoxycoethemoglobin. A supporting evidence is that the pH of unbuffered CoHbA in 1.0 M NaCl is 6.82 compared to 7.33 for FeHbA under the same conditions.

Among the eight salt bridges postulated to exist in FeHb (4) the ones between the imidazolium group of hisHC3(146)p, and aspFG1(94)p, are most readily subjected to scrutiny. A nitrooxide spin label attached to cys(93)p provides a most sensitive probe for the environment in its vicinity. Its EPR spectrum reveals that the mobility of the spin label becomes less restrained when the enzyme is oxygenated (18). This supports the idea that the above mentioned salt linkage is disrupted in the oxy state. The EPR spectra of CoHbA (Fig. 10a) shows that there is little restriction to the local motion of the spin label in this species. The label in this protein has even more mobility than it has when attached to FeHbA O_2. We take this to mean that there is no His-Asp linkage in the p chains of the cobalt species. The fact that the spin label EPR spectrum is unchanged by oxygenation should not be construed as there is no conformational changes in the vicinity of the label. The label has so much free space to move around in the deoxy state that any increase of this space in the oxy state cannot further reduce the EPR line widths.

The reaction rates with p-MB are entirely consistent with the
spin label data. The reaction rates are faster for CoHbA and CoHbO2 than for FeHbA and FeHbO2 indicating that the site of reaction is more accessible in the cobalt species. The small difference in the rate constants could mean that the environment in the vicinity of Cys(93)β is slightly less restrictive in CoHbO2 than it is in FeHbA.

The orientations of the heme plane in FeMb and CoMb are the same within the experimental accuracy of EPR (2). The same appears to be true in FeHbA and CoHbA (17). Therefore, it is probable that the COOH-terminal histidine has been moved to a new position by cobalt substitution. This displacement could conceivably sever the αβ salt bridges between the α-carboxyl group of HisHC3(146)β and the ε-amino group of LysC5-(40)α as well. Experiments are underway to determine the number of salt bridges undisrupted by cobalt substitution.

The spin label and p-MB results have another significant bearing on the Bohr effect. It may be said that the 1.0 M NaCl condition used in differential titration weakens the electrostatic interactions which are necessary to maintain the salt linkages. This of course does happen to a degree. However, the spin label and p-MB experiments were done under "normal" conditions. Therefore, there must be some conformational alterations in the CoHbA which are manifested in a reduction of salt bridges.

Let us turn our attention to the metal atom itself. In the deoxy protein the cobalt differs from iron in two important respects: it has a low spin configuration and its de axial bond orbital is singly occupied. In the low spin configuration its ionic radius should be sufficiently small to permit the cobalt atom to fit into the "central hole" of the porphyrin (3, 24) and is probably not located significantly out of the porphyrin plane. The de electron tends to repel axial ligand. The likely characteristic features of CoHbA are thus the nearly in-plane disposition of the cobalt nucleus and a rather long Co-N axial bond distance.

CoHbO2 is also in a low spin configuration. The unpaired electron is largely transferred to the oxygen as judged from the greatly reduced 58Co hyperfine splitting (1, 2) compared to that for the deoxy state. In fact there is accumulated a body of evidence supporting the view that the oxygen ligand in CoHbO2 can be thought of as a superoxide ion. The principal values of the g-tensor are very close to the 0− ion (1, 2). The O−O bond distances range from 1.26 A to 1.42 A in Co[NN'-ethylenebis(benzoylacetonimine)pyridine]O2 (25) and oxygen-bis[α-1,2-bis(diphenylphosphino)-ethylene]cobalttetrafluoroborate (26), respectively. These distances are to be compared to 1.21 A for O2 and 1.28 A for O2−. Furthermore, the optical spectrum of CoHbO2 suggests that the oxidation state of Co is closer to +3 than to +2 state (Figs. 2 and 3). With the unpaired electron delocalized to O2, the Co−N axial bond distance in CoHbA+ may be considerably shorter than it is in CoHbA. It is likely that contraction of this bond is primarily responsible for the "triggering" of the homotropic allosteric interaction.7 Judging from the cooperativity of CoHbA this contraction could be about 0.4 to 0.5 A.

The above hypothesis tends to modify somewhat the generally accepted triggering mechanism for the native enzyme. In the elegantly described stereochemical model of Perutz (4) the change from the high spin iron to the low spin iron causes a 0.75 Å shift of the metal atom from an out-of-plane position to an in-plane geometry initiating the allosteric transition. The Fe-N axial distance remain the same in this process. Large positional change of the metal atom per se appears to be a sufficient but not necessary condition. But rather large positional change of the proximal histidine is the necessary and sufficient condition for allosteric transition.

The oxidation-reduction measurements are particularly interesting. Aquated Co(II) ion is much more resistant to oxidation than the Fe(II) ion. The oxidation reduction potentials for the Co(III)/Co(II) and the Fe(III)/Fe(II) couples are +1.82 volts and +0.77 volt respectively. It has also been found that Co(II) complexes in solution, unlike the Fe(II) complexes, are sufficiently oxidation-resistant to exhibit reversible binding of molecular oxygen (26, 27). Nature compensates for the oxidative lability of hemoglobin with methemoglobin reductase to recycle the enzyme. It is valid to inquire whether the oxidative stability of the Co(II) complexes is retained in the cobalt protein. Our results show that, if anything, CoHbA is more easily oxidized than FeHbA. The near equality of the oxidation-reduction potential of the two proteins suggest that the porphyrin moiety and globin environment almost completely determine E1/2 and that E1/2 is an index of oxygen reversibility.

In conclusion, CoHbA shares many of the biological properties of the native enzyme. It is, however, an inferior oxygen carrier with diminished proton and COa linkages. Organic phosphates are less able to control the oxygen affinity of CoHbA than FeHbA because they form stable complex in the former. CoHbA has lower oxygen affinity and oxidative stability than the native species.

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