Ligand Binding to Cytochrome *c* and Other Related Haem Proteins and Peptides. Part II. Kinetic Studies

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(Received February 13, 1988)

Abstract

The kinetics of ligand binding to native cytochrome *c* and myoglobin seem to suggest that both proteins bind exogenous ligands by an S_N2 mechanism, while the form of cytochrome *c* which lacks the 695 nm absorption band binds ligands by a limiting S_N1 mechanism. It is suggested that the rate-limiting step in the S_N2 mechanism is different from that in the S_N1 mechanism.

Introduction

A characteristic property of N-cytochrome *c** is the sluggishess of its reactions with iron ligands [1–3]. Thus the binding of cyanide, although strong, is very slow [4]. The formation of complexes between N-cytochrome *c* and iron ligands is directly opposed by the iron–Met 80 bond [5]. Since the strength of this bond depends on the overall conformation of the protein [6], cm-cytochrome *c** and H-6-P* provide interesting models for studying the interaction between protein conformation and iron–ligand bonding.

Cyanide binding rates and equilibrium association constants with N-cytochrome *c* and modified cytochrome *c* may be used, in a comparative fashion, as a benchmark for haem accessibility in various forms of cytochrome *c* [7]. Cyanide was chosen (as the most extensively investigated ligand in this study) for several reasons: it induces large spectral changes on binding; it can be easily manipulated over wide concentration ranges; and it has high affinity for haem proteins. The affinity constants and the rates of cyanide binding to haem proteins are greatly influenced by the conformations of the proteins [7–9]. The thermodynamic parameters for hydrocyanic acid ionization are available [10], therefore it is possible to study the temperature dependencies of the affinity and rate constants. Thus, cyanide binding was used as a facile probe of the general haem environment and, when appropriate, data from the binding of other ligands were used.

The conclusions regarding the haem environment derived from such ligand binding studies are compared with the known structures of the haem sites as determined by X-ray crystallography and other techniques (for reviews see refs. 6, 11–13).

Experimental

The materials used are reported in the previous paper of this series [14]. A Durrum–Gibson stopped-flow instrument with a 2-cm light path and a dead-time of 3 ms was used for measuring the rates of exogenous ligands binding to the haem proteins and peptides used in this study. The reaction mixtures contained *ca.* 30 μM of the cytochrome *c* or *ca.* 20 μM of H-6-P and at least a 100-fold excess of the ligand used.

Results

Kinetic Binding Studies

Rapid kinetic experiments in which H-6-P was mixed with potassium cyanide under pseudo-first-order conditions led to a fast ligand concentration-dependent process, which at pH 7.5 was followed by a slower concentration-independent process. The rate of the fast phase was pH dependent, increasing from 4.47×10^4 M^{-1} s^{-1} at pH 7.5 to about 2.5×10^5 M^{-1} s^{-1} at pH 8.5. The rate of the slow phase was almost pH invarient (the rate at pH 7.5 was 0.43 s^{-1} and at pH 10.5 was 0.4 s^{-1}). The amplitudes of the two phases seen with H-6-P were pH dependent. The fast phase

*Abbreviations: N-cytochrome *c* = native cytochrome *c*; cm-cytochrome *c* = carboxymethyl-Met 80 cytochrome *c*; H-6-P = haemhexapeptide, residues 14–19 of native cytochrome *c*; H-11-P = haemundecapeptide, residues 11–21 of native cytochrome *c*.

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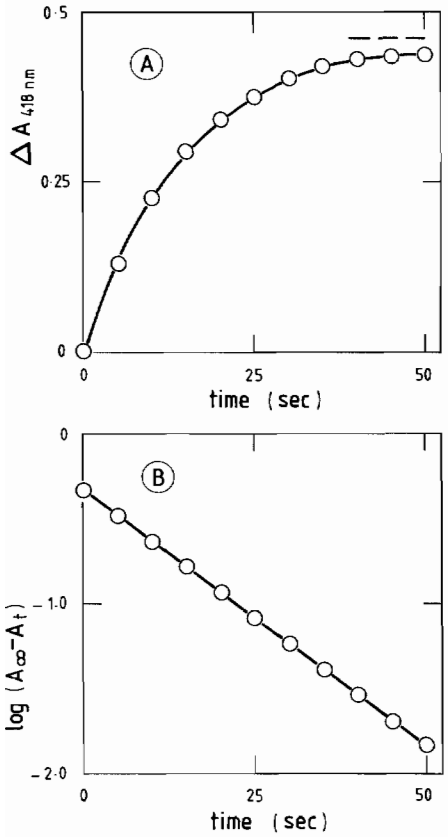

Fig. 1. Binding of cyanide to N-cytochrome *c* at 25 °C, pH 7.5 in 0.15 M phosphate buffer, followed in the stopped-flow method at 418 nm. Cyanide concentration after mixing was 9.5×10^{-2} M. (A) Shows the progress curve of the absorbance change at 418 nm *versus* time for the binding of cyanide to N-cytochrome *c*. (B) Shows the logarithm of the absorbance change upon cyanide binding to N-cytochrome *c* *versus* time.

amounted to about 80% at pH 7.5, while at pH 10.5 the fast phase completely disappeared and only the slow phase was detectable. These findings are in agreement with the results reported by Wilson *et al.* [15] on the binding of cyanide to cytochrome *c* haemundecapeptide.

Figure 1A shows a progress curve for the binding of 9.5×10^{-2} M cyanide to N-cytochrome *c* at pH 7.5. The binding conforms to a simple exponential, as shown in Fig. 1B. cm-Cytochrome *c* gave a similar binding progress curve to that of N-cytochrome *c* at this pH. The progress curves for cyanide binding to N-cytochrome *c* and cm-cytochrome *c* at pH 10.5 were also simple exponentials.

The pseudo-first-order rates as a function of cyanide concentration for the binding of cyanide to N-cytochrome *c* at pH 7.5 and pH 10.5 are shown in Fig. 2A. At pH 7.5 the pseudo-first-order rate was linearly dependent on cyanide concentration, up to 1.25 M cyanide, with no evidence of a limiting rate at

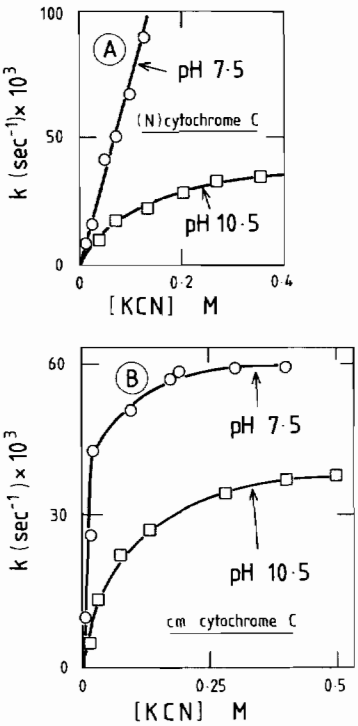

Fig. 2. Cyanide concentration dependence of the binding of cyanide to: (A) N-cytochrome *c* at pH 7.5 in 0.15 M phosphate buffer and at pH 10.5 in 0.15 M borate buffer at 25 °C; (B) cm-cytochrome *c* at pH 7.5 and pH 10.5 (conditions as in (A)).

a temperature of ≦ 50 °C. At temperatures greater than 50 °C, however, a limiting rate was observed. A value of 0.72 M^{-1} s^{-1} for the second-order rate constant for cyanide binding to N-cytochrome *c* at pH 7.5 and 25 °C (from Fig. 2A) is obtained. At pH 10.5, the plot of the pseudo-first-order rate constants against cyanide concentration for cyanide binding to N-cytochrome *c* shows a limiting rate of about 0.04 s^{-1}. The initial rate, determined from Fig. 2A, was 0.4 M^{-1} s^{-1}. Table I lists the rates of cyanide binding to N-cytochrome *c* and other haem proteins.

The pseudo-first-order rate for cyanide binding to cm-cytochrome *c* at pH 7.5 and 10.5 is shown in Fig. 2B. At both pH values a limiting rate is observed. The rate-limiting step at pH 7.5 has a value of about 0.06 s^{-1}, while at pH 10.5 it is about 0.04 s^{-1}. The initial rate of the pseudo-first-order plot at pH 7.5 yields a value of 5.0 M^{-1} s^{-1}, while at pH 10.5 a value of 0.69 M^{-1} s^{-1} is obtained.

Discussion

The binding of cyanide to N-cytochrome *c* at pH 7.5 (Fig. 2A) may be described by eqn. (1)

$$k_{obs} = k_a[KCN] + k_d \quad (1)$$

TABLE I. Second-order Rate Constants for Cyanide Binding to Haem Proteins (at 25 °C)

Protein	Ligand	pH	k (M^{-1} s^{-1})
N-Cytochrome *c*	cyanide	7.5	0.73
cm-Cytochrome *c*	cyanide	7.5	5.00
H-6-P	cyanide	7.5	4.47×10^4 [a]
Methmyoglobin	cyanide	7.5	2.24×10^2 [b]
N-Cytochrome *c*	cyanide	10.5	0.4
cm-Cytochrome *c*	cyanide	10.5	0.69
H-6-P	cyanide	10.5	0.4 [a]
Methmyoglobin	cyanide	10.5	4×10^2 [b]
H-6-P	cyanide	7.0	1.15×10^4 [b]
H-6-P	cyanide	8.5	2.5×10^5 [a]
N-Cytochrome *c* [c]	azide	7	14.2
N-Cytochrome *c* [c]	imidazole	7	26.7
N-Cytochrome *c* [d]	imidazole	9	22
Myoglobin [b]	azide	7.0	7×10^3
Myoglobin [b]	imidazole	7.0	2.4×10^2

[a] The fast-phase rate. [b] Calculated from ref. 16. [c] Calculated from ref. 2. [d] From ref. 19.

where k_{obs} is the observed rate constant and k_a and k_d are the association and the dissociation rate constants, respectively. The mechanism of cyanide binding to cytochrome *c* has been discussed by Margoliash and Schejter [5] in terms of an S_N2 mechanism. In the transition state of the reaction, a bond is formed between the iron and cyanide before the iron–Met 80 bond is entirely broken. The height of the activation barrier for this transition state may be interpreted in terms of distortion in the haem environment which depends upon the strength of the iron–Met 80 bond and the equilibrium between the open and closed structures of the haem crevice [14]. However, Sutin and Yandell [2] preferred the interpretation of a limiting S_N1 mechanism for the ligand binding data of cyanide, azide, imidazole and pyridine to N-cytochrome *c* at neutral pH. This requires an open crevice structure in N-cytochrome *c* for the binding to take place. Therefore, both mechanisms emphasize the importance of the haem crevice in the reactions of cytochrome *c* with exogenous ligands.

We now discuss our results in terms of the haem crevice equilibrium, the nature of the ligand binding kinetics of the protein, and the properties of the ligand. From Fig. 2A it is clear that the kinetics of cyanide association to N-cytochrome *c* at pH 10.5 is different from cyanide binding at pH 7.5 (a limiting rate of about 0.04 s^{-1} being reached at pH 10.5), since the binding of cyanide to the protein at pH 7.5 does not show a limiting rate up to cyanide concentrations of 1.25 M. We will consider first the second-order rate constants reported in Table I and subsequently discuss the limiting rate process.

At pH 7.5 the rate of cyanide binding to H-6-P is about five orders of magnitude greater than the cyanide association rate to N-cytochrome *c* (Table I). This is in accordance with the 'direct' binding site in H-6-P and the formation of complexes without steric hindrance. The closed crevice of N-cytochrome *c* and the steric effects resulting from cyanide ligation adequately account for the slow association of cyanide to N-cytochrome *c*. This is supported by cyanide association to cm-cytochrome *c* at the same pH, where the second-order rate for cyanide binding is about an order of magnitude faster than for the native protein. This indicates that the haem crevice in cm-cytochrome *c* is more open than in the native protein, but only partially so. The H-6-P rate is only about two orders of magnitude greater than the cyanide association rate to metmyoglobin at pH 7.5 (Table I). This is in accordance with the open haem pocket of myoglobin and the hydrophobic nature of this pocket, which because of its lower dielectric constant increases the coulombic attraction of the cyanide anion to the cationic haem iron. The rate enhancement for cyanide binding due to the haem pocket hydrophobicity of N-cytochrome *c* and cm-cytochrome *c* is severely offset by the relatively closed structure of their respective haem pockets. Myoglobin, however, seems to counteract the steric effect of cyanide ligation by virtue of its relatively open crevice.

Azide binding to metmyoglobin at pH 7.0 reflects the importance of the hydrophobicity of the haem pocket and the open site parameter, as azide ligation does not result in a steric effect on the protein [8, 9]. From Table I it is evident that the rate of azide binding to myoglobin is about an order of magnitude faster than cyanide binding to myoglobin, probably reflecting the effect of the steric factor in the rate of ligand association. This result is also observed with N-cytochrome *c*, where azide binding to N-cytochrome *c* is about an order of magnitude faster than cyanide binding to N-cytochrome *c*. Also the rate of azide binding to myoglobin at pH 7 is about two orders of magnitude greater than the rate of azide binding to N-cytochrome *c*, which reflect the 'open–closed' site parameter. The importance of this parameter is more evident in the rate of imidazole binding to metmyoglobin and N-cytochrome *c* at pH 7. The association constant for imidazole binding to myoglobin is about an order of magnitude faster than the corresponding rate for N-cytochrome *c* (Table I). It is interesting to note that the rate of azide binding to myoglobin at pH 7 is about 30 times faster than the rate of imidazole binding to myoglobin at the same pH. This is attributed to the size of the ligand, since imidazole is more bulky than azide and thus is sterically hindered from entering the haem pocket. This point is well illustrated by the rate of binding of alkyl isocyanide to myoglobin at pH 9.1 [16], the second-order rate decreased following this order: methyl (135×10^3 M^{-1} s^{-1}) > ethyl (65 ×

TABLE II. Limiting Rate Parameters for Cytochrome *c*

Protein	Ligand	pH	Temperature (°C)	k_1 (s^{-1})
N-Cytochrome *c*	cyanide	7.5	25	–
N-Cytochrome *c*	cyanide	7.5	51.5	2.35
N-Cytochrome *c*	cyanide	10.5	25	0.04
cm-Cytochrome *c*	cyanide	7.5	25	0.06
cm-Cytochrome *c*	cyanide	7.5	51.5	2.1
cm-Cytochrome *c*	cyanide	10.5	25	0.04
d-Cytochrome *c*	cyanide	7.2	25	0.06
N-Cytochrome *c*[b]	imidazole	7.4	12	0.49
N-Cytochrome *c*[c]	imidazole	9	21	8
N-Cytochrome *c*[d]	imidazole	7	25	60
N-Cytochrome *c*[d]	pyridine	7	25	58
N-Cytochrome *c*[d]	azide	7	25	30
N-Cytochrome *c*[e]	(alkaline isomerisation)	–	25	6.1

[a]d is abbreviation for derivation and the derivatives used were: iodinated cytochrome *c* at Tyn 67 and 74; formylated cytochrome *c* at Try 59; Met 80 sufoxide derivative of *N*-acyl isourea (modifying the haem propionate groups). From ref. 7. [b]From ref. 18. [c]From ref. 19. [d]From ref. 2. [e]From ref. 26.

10^3) > n-propyl (43×10^3) > isopropyl (17×10^3) > isobutyl (2×10^3) > tert-butyl (1.6×10^3). However, N-cytochrome *c* binds imidazole at a rate almost double the rate of azide (Table I). This apparent contradiction is explained in terms of the strength of the exogenous ligand used. Azide, as an intermediate-field ligand, will be less effective in replacing Met 80, an intermediate-to-strong-field ligand, compared to imidazole, which is itself an intermediate-to-strong-field ligand.

At high pH (>10), N-cytochrome *c* has a similar haem crevice to cm-cytochrome *c* [17]. Thus, the rate of cyanide binding to N-cytochrome *c* is comparable to the rate of cyanide binding to cm-cytochrome *c* (Table I). But the rate of cyanide binding to cm-cytochrome *c* is about an order of magnitude lower at this pH compared to the rate of cyanide binding to cm-cytochrome *c* at pH 7.5, in accord with the presence of a stronger endogenous ligand at high pH (>10) compared to neutral pH, as discussed earlier. However, the rate of cyanide binding to N-cytochrome *c* at pH 10.5 is almost half the rate of cyanide binding at pH 7.5, in spite of the iron being most accessible [12] at high pH and the higher CN^- concentration. This lowering in rate is not drastic, but is of significance and this will be discussed later. For metmyoglobin the rate of cyanide binding at pH 10.5 is almost double the rate at pH 7.5 (Table I), which is in agreement with greater haem accessibility ('open–closed' haem pocket parameter).

Figure 2 shows the limiting rates for N-cytochrome *c* at pH 10.5 and cm-cytochrome *c* at pH 7.5 and pH 10.5 in their reaction with cyanide. This rate-limiting process is reported in the literature [2, 18, 19], but without agreement on the value of this rate, as seen in Table II. The work of Dyer *et al.* [7] on modified cytochrome *c* yields a value of 6×10^2 s^{-1} for cyanide binding to various cytochrome *c* derivatives, which is in excellent agreement with the value obtained for cyanide binding to cm-cytochrome *c* at similar pH values (Table II). These workers found no pH dependence in the rate of cyanide binding to various modified forms of cytochrome *c* in the pH range 7–9, indicating that the rate-limiting step does not depend on the protonation state of cyanide. This behaviour is in marked contrast to N-cytochrome *c*, where the rate of cyanide binding is first-order in total cyanide concentration and is strongly pH dependent [4]. The difference in the behaviour between N-cytochrome *c* and modified cytochrome *c* demonstrates that a new rate-limiting step has become operative on chemical modification. All the derivatives reported by Dyer *et al.* [7] (iodinated cytochrome *c* at Tyr 67 and 74; formylated cytochrome *c* at Trp 59; Met 80 sulfoxide derivative and the haem propionate *N*-acyl-isourea derivative) lack the 695 nm band, as does cm-cytochrome *c*. This band is thought to result from a charge transfer between the sulfur atom coordinated to the central haem iron. Thus it seems the rupture of the Met 80–iron bond results in the new rate-limiting step. This is supported by the similar behaviour of the alkaline from of cytochrome *c* to that of modified cytochrome *c* (Fig. 2A) and the rate of cyanide binding to N-cytochrome *c* at high temperatures (>50 °C) [20]. Both these forms of cytochrome *c* lack the 695 nm absorption band. Therefore, breakage of the Met 80–iron bond results in, among other things, loosening of the haem crevice [12], but from the kinetic evidence discussed above a deeper analysis of this process is required.

Rupture of Met 80 ligation to the haem iron allows Met 80 to swing away from the haem iron by rotating the polypeptide chain (residues 79–83) counter-clockwise, as viewed from the top of the molecule [21]. If this happens, then the ϵ-amino group of Lys 79 becomes positioned inside the haem crevice and is in proximity to the haem iron ligation site. Work by Bosshard [22] shows that the Lys 79 ϵ-amino group changes from being 'exposed' in N-cytochrome *c*, to a 'buried' position in the alkaline form of cytochrome *c*; this is also in agreement with the work of Mandel *et al.* [23]. This movement results in a breaking of the salt bridge (between Lys 79 and Thr 47) located at the bottom of the haem crevice [12]; this disrupts the net of hydrogen bonding at the bottom of the crevice [24] and leads to a more open crevice than found in the native protein. However, these movements result in a new barrier being produced to the approach of exogenous ligands to the haem iron. Such a barrier will be detected by ligand binding kinetics rather than ligand binding equilibria. Cytochromes *c* lacking the 695 nm band still perform a crevice 'breathing' process [3, 7], thus we expect this new barrier to be temperature and pH dependent and, indeed, this seems to be the case (see Table II). It appears, therefore, that there is a distinction between the rate-limiting step observed with N-cytochrome *c* exhibiting the 695 nm band and the rate-limiting step observed with cytochromes *c* lacking the 695 nm band. The former may be ascribed to an S_N2 mechansim, as described by Margoliash and Schejter [5], while the latter is controlled by the 'breathing' of the haem crevice and the movement of residues 79–82 to allow the exogenous ligand to approach the haem iron.

The mechanism by which cytochrome *c* lacking the 695 nm band binds exogenous ligands is represented as follows:

$$\text{d cyt } c \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{d cyt } c^* \qquad (2)$$

$$\text{d cyt } c^* + [\text{L}] \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{d cyt } c\text{–L} \qquad (3)$$

where d cyt *c* represents the cytochrome *c* lacking the 695 nm band and d cyt *c** represents a conformationally altered species reactive towards exogenous ligands (L). The observed pseudo-first-order rate is then given by

$$k_{obs} = \frac{k_1 k_2 [\text{L}] + k_{-1} k_{-2}}{k_1 + k_{-1} + k_2 [\text{L}] + k_{-2}} \qquad (4)$$

where $k_{-1} \gg (k_1 + k_{-2})$ is a reasonable assumption (see ref. 2).

Thus, the lower apparent second-order rate constant for cyanide binding to N-cytochrome *c* at pH 10.5 compared to cyanide binding at pH 7.5 is a reflection of this new barrier imposing a rate limit, as described above. The approximate 10-fold lower rate of cyanide binding to cm-cytochrome *c* at pH 10.5 compared with pH 7.5 (Fig. 2 and Table I) presumably indicates the pH dependence of this new barrier. The ionization rate of the ϵ-amino group of Lys 79 (with the intrinsic $pK_a = 10.2$ lower than the expected $pK_a = 11$ for this group [22]) may have a part to play in this pH dependence.

Similarly, an increase in temperature (25 to 51.5 °C) causing the polypeptide chain forming the haem crevice to unwind partially [25] and increasing the access of exogenous ligands to the haem leads to an enhanced rate of cyanide binding to cm-cytochrome *c* at pH 7.5.

The reactions between cytochrome *c* and azide or imidazole yield apparently conflicting data, especially in the evaluation of a rate limit (see Table II). However, some of these discrepancies may perhaps be rationalized. Sutin and Yandell [2] measured increasing rates of azide and imidazole binding to cytochrome *c* at pH 7 in the concentration range up to 0.5 M ligand (the pseudo-first-order rates being 11.8 and 14.6 s^{-1} respectively at 0.5 M ligand). The pseudo-first-order rates measured at 50 mM imidazole are, however, a factor of 2 higher than those reported by Greenwood and Palmer [18] and are similarly close to those measured, under somewhat different conditions by Al-Ayash and Wilson [19] at 0.3 M imidazole. The differences arise in the interpretation and extrapolation of these values to yield rate limits. Over the concentration range explored at pH 7, the rates do not clearly plateau and this may reflect an S_N2 mechanism for the binding, in agreement with the proposal of Margoliash and Schejter [5] and, in addition, may lead to very high apparent rate limits if treated by an S_N1 formulation (eqn. (4)), as reported by Sutin and Yandell [2] (Table II).

Thus we may rationalize the data in Table II by supposing an S_N2 mechanism for ligand binding to N-cytochrome *c* exhibiting a 695 nm band. According to such a mechanism, the rate-determining step is the ligand attack on the haem iron such that in the transition state a bond is formed between the exogenous ligand and the iron before the iron–protein bond is entirely broken. This is supported by cyanide binding (Fig. 2A and ref. 4) and azide and imidazole binding [2]. The strength of the Met 80–iron bond and the haem crevice are factors affecting the affinity and rate of ligand binding, but are not rate limiting. Thus the height of the activation barrier for the transition state may be interpreted in terms of the iron–protein bond and the 'open–closed' haem crevice parameter. This mechanism seems to be operating also in reac-

tions of myoglobin with ligands [5], which may have a physiological significance.

Al-Ayash and Wilson [19] reported a limiting rate value of 8 s^{-1} for imidazole binding to N-cytochrome *c* at pH 9. At this pH the protein exists in two intermediate forms, one exhibiting the 695 nm absorption band and one which does not. The value of 8 s^{-1} is in reasonable agreement with a value of 6.1 s^{-1} reported for the alkaline transition forward rate k_1 of eqn. (2) (at similar pH value) by Davis *et al.*, [26]. At pH 9, therefore, imidazole binding as reported by these workers may involve both S_N1 and S_N2 mechanisms. Further study of this system is required to elucidate this point.

In conclusion, the ligand binding data from this study and from the literature seem to suggest an S_N2 mechanism for ligand binding to cytochrome *c* exhibiting the 695 nm band and myoglobin, while cytochrome *c* lacking the 695 nm band binds ligands by a limiting S_N1 mechanism represented by eqn. (4).

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