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Purification of tryptophan containing synthetic peptides by selective binding of the α-amino group to immobilised metal ions☆

Per Hansen*, Gunnar Lindeberg

Department of Immunology, Biomedical Center, University of Uppsala, Box 582, S-751 23 Uppsala, Sweden

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Abstract

Immobilised metal ion affinity chromatography (IMAC) based on selective binding via the α-amino group to Cu^{2+} and Ni^{2+} ions has been used to purify tryptophan containing synthetic peptides. A free α-amino group, serving as an affinity handle, is present only in the target peptide when the peptides are synthesised by the solid-phase method and remaining amino groups after each coupling step are blocked by acetylation.

A free α-amino group is necessary to retain the peptide on the column. The tryptophan residue may contribute to the binding only if the peptide is simultaneously anchored via the α-amino group.

1. Introduction

Immobilised metal ion affinity chromatography (IMAC) was introduced by Porath *et al.* in 1975 [1] and has since been a useful tool for the purification of various proteins (*e.g.* refs. 2–11). The α-amino group is known to interact with immobilised metal ions [12–17], but it is generally assumed that it is histidine, cysteine and tryptophan that are largely responsible for the binding of proteins [1,18–20] and of peptides [15,16,21] in IMAC. Accordingly, these amino acids, when situated near the N-terminus, have been used as affinity handles for the purification by IMAC of synthetic peptides [22].

Crude peptides synthesised by the solid-phase method using a capping protocol contain, apart from the target peptide with its free α-amino function, truncated peptides with blocked α-amino groups. Affinity handles have been introduced on the free α-amino group of the target peptide [23–28] thus allowing the use of more specific separation methods than RP-HPLC, which is commonly used for purification of synthetic peptides. The limitations of this approach, and particularly the problems associated with the removal of the substituent, prompted us to investigate the use of the free α-amino group as an affinity handle [29]. We found that IMAC could be used for the purification, via the free α-amino group, of synthetic peptides lacking histidine, cysteine and tryptophan. Such peptides are retarded in IMAC only when a free α-amino group is present. The retention is pH dependent and increases from pH 5 to 7.5 (Cu^{2+}) and from pH 5 to 8.5 (Ni^{2+}) due to deprotonation of the

* Corresponding author.

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α-amino group. As the pH is further raised to 9–10 the retention drops as a result of metal ion transfer (MIT) from the chromatographic support to the peptide. At pH > 9 (on Cu^{2+}, but not Ni^{2+}) the retention of lysine containing peptides again increases due to deprotonation and binding of the ϵ-amino group.

We have now widened our study to determine if selective binding via the free α-amino group is also possible when a tryptophan residue is present in the peptide. Since tryptophan strongly contributes to the binding of peptides and proteins in IMAC [15,16,18,21,30,31], it could be expected that each peptide containing a tryptophan residue would bind to the IMAC adsorbent, thereby destroying the selectivity. In an attempt to reduce the presumed metal affinity of the indole function we decided to protect the indole nitrogen by formylation. However, such precautions proved to be unnecessary. Data presented in this report clearly show that the contribution by tryptophan to binding is of secondary importance and can be observed only when the peptide is simultaneously anchored to the IMAC support by a more efficient affinity handle such as the α-amino group.

2. Experimental

2.1 Chemicals

All chemicals were of analytical grade and used as purchased. *tert*-Butyloxycarbonyl (Boc) amino acids were obtained from Peninsula Laboratories Europe (St. Helens, UK) or Novabiochem (Läufelfingen, Switzerland). Boc-amino acyl resins were prepared according to Horiki *et al.* [32]. Chelating Superose was obtained from Kabi-Pharmacia (Uppsala, Sweden).

2.2 Buffers

The chromatographic buffers (pH range 5–11) contained 50 m*M* sodium dihydrogen phosphate, 50 m*M* boric acid and 1 *M* sodium chloride and were prepared as described previously [29].

2.3 Peptide synthesis

Solid-phase synthesis of peptides was performed on an Applied Biosystems 430A instrument as described previously [29]. Formylated tryptophan peptides were deprotected by reaction with 0.1 *M* aqueous piperidine at room temperature for 30 min, then desalted on a Sephadex G-10 gel filtration column (30 × 1 cm I.D.) equilibrated with 50 m*M* ammonium acetate pH 8.0 and recovered by lyophilization.

Spontaneous deformylation of peptides occurred in the upper pH range. After one week at pH 6.5 no deformylation was detected, at pH 8.5 partial deformylation was observed and at pH 10.0 peptides were completely deformylated. The peptide solutions were never prepared more than 8 h before use.

The peptides are listed in Table 1 and will hereafter be referred to in bold numbers with indication of a free (**A**) or blocked (**B**) α-amino group.

2.4 IMAC

An FPLC system (Kabi-Pharmacia, Uppsala, Sweden) was used for all chromatography and the conditions for IMAC were as described earlier [29]. The peptides (100 nmoles in 100 μl of the chromatographic buffer) were applied to the pre-equilibrated column and eluted isocratically. The capacity factor (k) was determined according to

$$k = V_e/V_0 - 1$$

where V_e is the retention volume for the peptide on a metal loaded column and V_0 the retention volume on a metal free column.

2.5 Mass spectrometry

Synthetic products and chromatographic fractions were analysed by plasma desorption mass spectrometry (PDMS) using a BioIon 20 instrument (Applied Biosystems, Uppsala, Sweden) as described earlier [29].

Table 1
Structures of peptides used in this investigation

Peptide	Structure
1A [a]	Gly–Ala–Thr–Lys–Gly–Pro–Gly–Arg–Val–Ile–Tyr–Ala
1B [a]	Ac–Gly–Ala–Thr–Lys–Gly–Pro–Gly–Arg–Val–Ile–Tyr–Ala
2A	Gly–Ala–Thr–Lys–Gly–Ile–Gly–Arg–**Trp**–Ile–Tyr–Ala
2B	Ac–Gly–Ala–Thr–Lys–Gly–Ile–Gly–Arg–**Trp**–Ile–Tyr–Ala
3A	Gly–Ala–Thr–Lys–Gly–Ile–Gly–Arg–**Trp**(For)–Ile–Tyr–Ala
3B	Ac–Gly–Ala–Thr–Lys–Gly–Ile–Gly–Arg–**Trp**(For)–Ile–Tyr–Ala
4A	Leu–Glu–Leu–Arg–Ser–Arg–Tyr–Val–Ala–Ile–Arg–Thr–Arg–Ser–Gly–Gly–NH_2
4B	Ac–Leu–Glu–Leu–Arg–Ser–Arg–Tyr–Val–Ala–Ile–Arg–Thr–Arg–Ser–Gly–Gly–NH_2
5A	Leu–Glu–Leu–Arg–Ser–Arg–Tyr–**Trp**–Ala–Ile–Arg–Thr–Arg–Ser–Gly–Gly–NH_2
5B	Ac–Leu–Glu–Leu–Arg–Ser–Arg–Tyr–**Trp**–Ala–Ile–Arg–Thr–Arg–Ser–Gly–Gly–NH_2
6A	Leu–Glu–Leu–Arg–Ser–Arg–Tyr–**Trp**(For)–Ala–Ile–Arg–Thr–Arg–Ser–Gly–Gly–NH_2
6B	Ac–Leu–Glu–Leu–Arg–Ser–Arg–Tyr–**Trp**(For)–Ala–Ile–Arg–Thr–Arg–Ser–Gly–Gly–NH_2

[a] Taken from ref. 29.

2.6 Trypsin digestion

Chromatographic fractions from IMAC were concentrated and desalted on a 10 × 1 cm I.D. Pep-RPC column. After adsorption, the column was washed with 0.1% aqueous TFA and the peptides eluted with a steep acetonitrile gradient (0–60% in 1 min). The material was recovered by lyophilization and then dissolved in 0.2 ml of 50 m*M* Tris–HCl, 50 m*M* glycine, pH 8.9. Trypsin (50 μg) was added and after 15 min at 37°C, samples were removed for PDMS analysis, none of the original material being detected after this time.

3. Results and discussion

Analogues of peptide **1** were synthesised with the Val in position 9 replaced with Trp (peptide **2**) or Trp(For) (peptide **3**). Peptides **5** and **6** were similarly derived from peptide **4**. The effect of the tryptophan residue on the retention of the peptides on Cu^{2+}- and Ni^{2+}-loaded columns was investigated (Fig. 1 and 2). The N-terminally blocked peptides **1B–6B** showed no retention on either metal over the entire pH range, with the exception of the lysine containing peptides **1B–3B** which were retained on Cu^{2+} above pH 9. That peptides **2B** and **5B** with unprotected tryptophan were not retained was surprising, challenging earlier assumptions about the importance of tryptophan in the binding of proteins and peptides in IMAC [15,16,18,21,30,31].

Peptides **1A–6A** with a free α-amino group showed increasing retention from pH 5 to 7.5 (Cu^{2+}) or pH 5 to 9 (Ni^{2+}), and decreasing retention from pH 7.5 to 9 (Cu^{2+}) or pH 9 to 9.5 (Ni^{2+}). Only the lysine containing peptides **1A–**

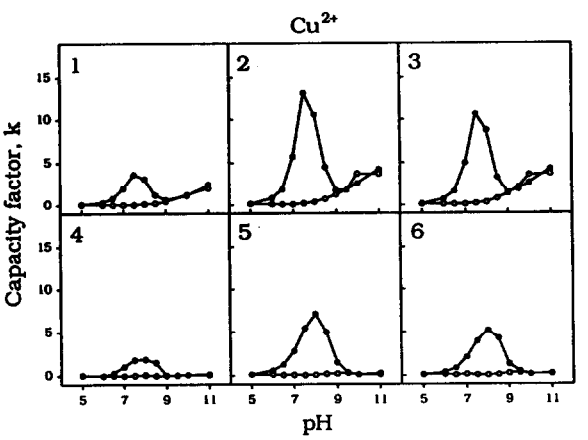

Fig. 1. Capacity factors *vs.* pH of peptides **1–6** on immobilised Cu^{2+}. The number in each square corresponds to the number of the peptide investigated (Table 1). Peptides with free α-amino groups are indicated with ● and blocked with ○. Column, Chelating Superose (1.8 × 1 cm I.D.) charged with Cu^{2+}. Elution, isocratic with 50 m*M* sodium phosphate/borate, 1 *M* NaCl at 1 ml/min. Sample, 100 nmoles of peptide dissolved in 100 μl of chromatographic buffer. Detection, UV at 280 nm.

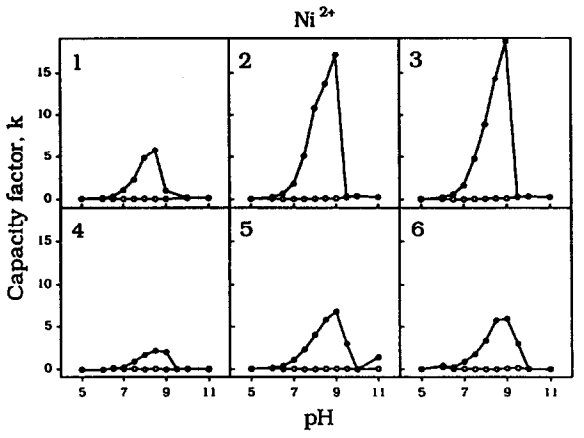

Fig. 2. Capacity factors *vs.* pH of peptides **1–6** on immobilised Ni^{2+}. Indications and chromatographic conditions as in Fig. 1.

3A were retained at pH 9–11 (Cu^{2+}). These findings suggest that the binding occurs by the mechanism proposed earlier [29], where the peptide is initially bound via the α-amino group and the neighbouring amide oxygen.

The tryptophan peptides **2A** and **3A** are structurally similar to peptide **1A** but their capacity factors are considerably higher (Fig. 1.1–1.3 and Fig. 2.1–2.3). In contrast, tryptophan contributes only moderately to the binding of peptides **5A** and **6A** (Fig. 1.4–1.6 and Fig. 2.4–2.6).

We can conclude from the results above that at least in the case of His- and Cys-free peptides a free α-amino group is necessary for binding to immobilised metal ions. The presence of a tryptophan residue in the sequence is in itself not sufficient for binding but can contribute in what seems to be a sequence dependent manner once the peptide has been anchored via the free α-amino group. It may be that other functional groups such as the imidazole of histidine could also serve as primary anchor points.

The capacity factors of peptides **2A** and **5A** are almost identical with those of the formylated peptides **3A** and **6A**, respectively, and not, as might be expected, significantly higher (Fig. 1 and Fig. 2). The increased retention is therefore unlikely to be caused by the indole nitrogen of the tryptophan residue complexing with a metal ion. The interaction could instead be hydrophobic in nature where the non-polar tryptophan side-chain interacts with the chromatographic support [33,34] or of charge-transfer character where the interaction occurs between the aromatic electrons of tryptophan and the metal ion [30]. The effect is only observed once the peptide has been anchored through the free α-amino group.

To confirm that the indole nitrogen is not contributing to the binding of peptides **2A**, **3A**, **5A** and **6A**, tryptamine and indole were included in the investigation. Tryptamine was used instead of tryptophan, since it is known that all amino acids chelate strongly as bidentate ligands to metal ions [12,16,35]. The binding of tryptamine to immobilised metal ions shows the typical pH dependence due to deprotonation of the amino group (Fig. 3). In addition there is a non-specific interaction with the chromatographic support causing *ca.* 2.5 times higher retention times on a metal free column than for peptides **1–6**. Indole, on the other hand, is retained in a pH independent manner equally on both metal loaded and metal free columns, with *ca.* 10-fold higher retention times than for peptides **1–6**. As in Fig. 1 and Fig. 2 the nitrogen of the indole is not seen to contribute to the binding over the pH range investigated. On the other hand, the metal independent binding of indole and tryptamine would suggest that the binding is due to non-specific hydrophobic interactions rather than charge transfer effects. However, when the indole function is present in a peptide these interactions are not as pronounced (only slightly higher retention times on a metal free column

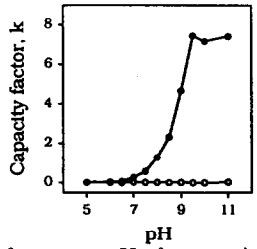

Fig. 3. Capacity factors *vs.* pH of tryptamine (●) and indole (○) on immobilised Cu^{2+}. Chromatographic conditions as in Fig. 1. On a Ni^{2+}-loaded support the capacity factors are close to zero over the entire pH range.

than for non-tryptophan containing peptides) and only contribute to the binding of a peptide that is already anchored to the matrix via a free α-amino group.

Yip *et al.* [15] studied the adsorption properties of several synthetic, biologically active peptides on IMAC columns. Two of these are of interest in the context of this report: human gastrin I (<Glu – Gly – Pro – Trp – Leu – Glu – Glu – Glu – Glu – Glu – Ala – Tyr – Gly – Trp – Met – Asp – Phe – NH_2) which despite the presence of two tryptophan residues is only slightly retarded at pH 7 and somatostatin (Ala – Gly – Cys – Lys – Asn – Phe – Phe – Trp – Lys – Thr – Phe – Thr – Ser – Cys) which shows a normal retention. These results support our findings that a peptide with a free α-amino group (somatostatin) will be bound in IMAC, while a peptide with a blocked α-amino group (human gastrin I) will not. In earlier purifications of synthetic cecropin A and a cecropin–mellitin hybrid the separation achieved was ascribed to the presence of a tryptophan residue in the N-terminal part of the sequence [22]. However, in view of the results presented here it seems that binding via the α-amino function, possibly amplified by hydrophobic interaction of the indole group, would provide a more likely explanation for the resolving power.

The usefulness of IMAC for the isolation of synthetic peptides via interaction of the α-amino group with an immobilised metal ion is demonstrated in the purification of the tryptophan containing peptide **2A**. The peptide was synthesised with formyl protected tryptophan, then the crude mixture subjected to deformylation as described above (Experimental). The crude peptide was applied on Ni^{2+}-loaded Superose and eluted with an increasing gradient of NH_4Cl at pH 8.5 (Fig. 4A). Two major fractions were obtained, which were further analysed by HPLC and mass spectrometry (Fig. 4B–E). As expected, peptide **2A** was detected in the main fraction (4A-II), together with a small amount of

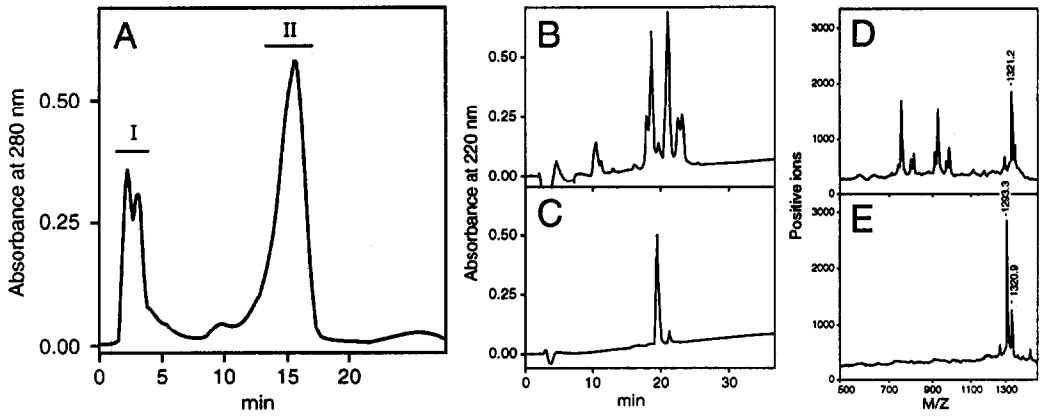

Fig. 4. (A) Chromatography of crude peptide **2A** on Chelating Superose-Ni^{2+}. Column dimensions, 1.8 × 1 cm I.D. Sample, 1.25 mg dissolved in 250 μl of 50 m*M* sodium phosphate/borate, 1 *M* NaCl, pH 8.5. Elution, 30-min gradient from 0 to 0.5 *M* NH_4Cl in the same buffer at 1 ml/min. For mass spectrometry the fractions indicated were concentrated and desalted on a 5 × 0.5 cm I.D. Pep-RPC column. After adsorption, the column was washed with 0.1% aqueous TFA and the peptides eluted with a steep acetonitrile gradient (0–60% in 1 min). (B) RP-HPLC of 1.5 ml (40%) of fraction 4A-I. Column, Pep-RPC (5 × 0.5 cm I.D.). Flow rate, 1 ml/min. Solvent A, 0.1% aqueous TFA. Solvent B, 0.1% TFA in acetonitrile. Gradient, 0–40% B in 30 min. (C) RP-HPLC of 200 μl (5%) of fraction 4A-II. Chromatographic conditions as in Fig. 4B. (D) PDMS of fraction 4A-I. The desalted sample (5 μl) was mixed with ethanol (2 μl) on a nitrocellulose coated aluminium foil, dried and rinsed with distilled water (20 μl). Most peaks can be attributed to various acetylated peptides and their fragment ions. The peak at 1321.2 mass units is due to migration of the formyl protecting group from the tryptophan residue to the α-amino group during deprotection. (E) PDMS of fraction 4A-II. Conditions as in Fig. 4D. The calculated molecular weight for peptide 2A is 1292.5. The peak at 1320.9 mass units is due to migration of the formyl protecting group from the tryptophan residue to the ϵ-amino group during deprotection.

formylated peptide presumably resulting from incomplete deprotection. Truncated peptides that had been acetylated in the capping steps were present in the non-retarded material (4A-I) together with a peptide of the same molecular weight as the formylated peptide **3A**. This surprising result could be explained by rearrangement of the formyl group from the tryptophan residue to the α-amino group during deprotection [36].

The following experiment was conducted to confirm that fraction 4A-I only contained peptides with blocked α-amino groups and that the anomalies mentioned above are artefacts of the deformylation procedure. The crude formylated peptide **3A** was applied to Ni^{2+}-loaded Superose and eluted with an increasing gradient of NH_4Cl at pH 8.5. The main fraction containing purified peptide **3A** was desalted and concentrated by RP-HPLC and then deformylated as outlined previously except that the sample was not desalted by gel filtration but diluted with 5–10 volumes of water prior to freeze drying. The deformylated material was then again applied on Ni^{2+}-loaded Superose (Fig. 5A) and the two major fractions analysed (Fig. 5B–E). As can be seen in Fig. 5, fraction 5A-II contains the expected product **2A** as the main component but also a small amount of formylated peptide. A formyl peptide is present also in fraction 5A-I. Tryptic digestion and PDMS analysis of fraction 5A-I and 5C-I (Table 2) revealed that neither peptide contained Trp(For). The formyl group must therefore have been relocated, either to the α- or to the ϵ-amino group. Since trypsin is unable to cleave the Lys–Gly bond in fraction 5C-I we may conclude that the ϵ-amino group is

Table 2
Trypsin digestion of GATKGIGRWIYA (**2A**)

Peptide fragment	Fraction 5A-I	Fraction 5C-I
GATK	For [a]	–
GIGR	+ [a]	–
WIYA	+	+
GATKGIGR	–	For
GIGRWIYA	–	–

The purified peptide GATKGIGRW(For)IYA was deformylated and separated using IMAC (Fig. 5A). The fractions 5A-I and 5C-I, both containing a peptide with an extra formyl group, were desalted and concentrated by RP-HPLC and then evaporated to dryness. The residues were dissolved in 0.2 ml of 50 m*M* Tris–HCl, 50 m*M* glycine, pH 8.9, and trypsin (50 μg) was added. After 15 min at 37°C the digests were analysed by PDMS.

[a] "+" indicates that a mass peak corresponding to the expected molecular weight was observed, "For" that the mass was 28 units higher than calculated.

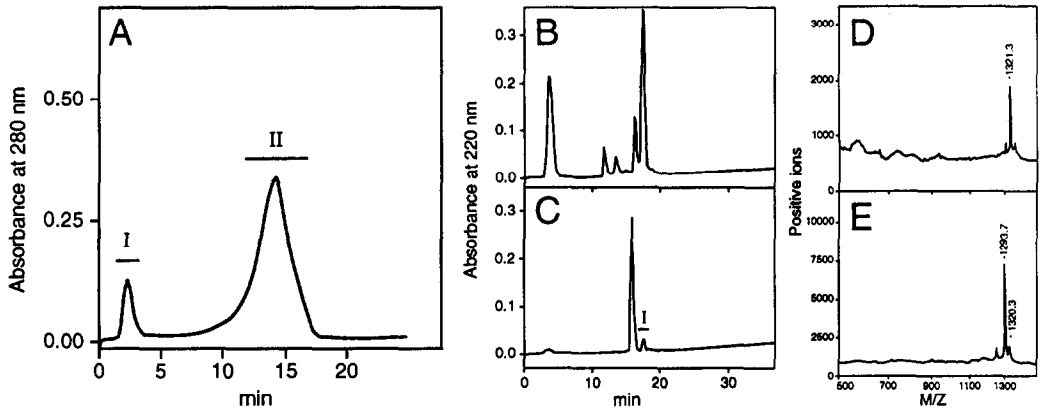

Fig. 5. (A) Chromatography of purified and deformylated peptide **3A** on Chelating Superose-Ni^{2+}. Chromatographic conditions as in Fig. 4A. (B) RP-HPLC of 1.2 ml (80%) of fraction 5A-I. Chromatographic conditions as in Fig. 4B. (C) RP-HPLC of 240 μl (4%) of fraction 5A-II. Chromatographic conditions as in Fig. 4B. (D) PDMS of fraction 5A-I. Conditions as in Fig. 4D. The peak at 1321.3 mass units is caused by rearrangement of the formyl protecting group to the α-amino function. (E) PDMS of fraction 5A-II. Conditions as in Fig. 4D. The calculated molecular weight for peptide **2A** (deformylated peptide **3A**) is 1292.5. The peak at 1320.3 mass units is due to migration of the formyl group to the ϵ-amino group.

modified (formylated). In fraction 5A-I the lysyl bond is cleaved in the normal way. In this case, the formyl group is most likely present as an α-amino substituent which explains why the peptide is not retarded on the IMAC support.

4. Conclusion

We have shown that IMAC based on metal binding via the α-amino group can be used for selective purification of synthetic peptides even when a tryptophan residue is present in the sequence. The target peptide with its free α-amino group will be well separated from the truncated peptides. The tryptophan residue alone is not sufficient for binding to immobilised metal ions; an effect may be observed only when the peptide is first anchored by the α-amino group. A particular advantage of this affinity method is that no modification of the target peptide is necessary. Consequently, material losses accompanying the introduction and removal of a specific affinity handle are avoided and the solubility is not affected.

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