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An unusual metal-binding cluster found exclusively in the avian breast muscle troponin T of *Galliformes* and *Craciformes*

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Abstract

A repeating metal-binding ($Cu^{2+} > Ni^{2+} > Zn^{2+} \sim Co^{2+}$) sequence $(HE/AEAH)_4$ has been identified in troponin T isoforms specifically expressed in the breast but not leg muscles of all *Galliformes* and *Craciformes*. It is absent in the skeletal and cardiac muscles of mammals and all other avian species investigated. Concentration of the metal-binding sites is adequate to affect free metal levels in the muscle cell and we suggest a possible link between its presence in breast muscle of *Galliformes* and the high ratio of breast muscle to total body muscle mass and explosive but short-lived flight pattern of these birds. This sequence can be used for a highly selective metal-affinity chromatographic purification of muscle or engineered TnTs even in high salt and/or urea.

Key words: Metal-binding cluster; Troponin T; Zinc; *Galliformes*; Breast muscle; Affinity purification

1. Introduction

Troponin T (TnT), the tropomyosin-binding subunit of the troponin complex, is a major protein in vertebrate striated muscles and participates in the Ca^{2+}-dependent regulation of contraction [1]. While different genes encode the cardiac and skeletal muscle TnTs, the expression of various isoforms from either gene is regulated through developmental and/or muscle type-specific alternative mRNA splicing involving as many as six exons corresponding to the hypervariable NH_2-terminal region of the TnT sequence [2–6]. Although the alternative splicing generated switch of cardiac TnT isoform expression appears to be well regulated during both avian and mammalian heart development [3,7,8], the functional significance of this hypervariable region has not been established. All of the alternatively spliced exons in this region encode highly acidic protein sequences and their expression in different combinations contribute to the wide isoelectric point range of the TnT isoforms. In chicken breast muscle, both cDNA [4] and protein [9] sequencing have demonstrated a segment consisting of four repeated histidine pairs (HEEAHHEEAHHAEAHHEEAH) in at least one major TnT isoform (see Fig. 1). This sequence (designated Tx) is apparently largely encoded by an alternatively spliced exon(s) (designated X) of the chicken skeletal muscle TnT gene [4]. To date, it has not been observed in protein or DNA sequences of TnTs from any other source [2,3,5,6,10–17]. Secondary structure predictions suggest that the $[HE/AEH]_4$ sequence in the chicken TnT1 isoform may exist in the å-helical form.

Recent studies [18] have demonstrated that a pair of histidine residues, separated by three amino acid residues (HXXXH) in an α-helix, forms a high-affinity binding site for such transition metal ions as Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+}. Construction of an HXXXH sequence in an engineered protein has been used as an approach for selective purification by metal-affinity chromatography [19]. Similar zinc-binding sites in the three-dimensional structures of thermolysin [20] and carboxypeptidase [21] have been described. In both cases these involve the side chains of two histidines and one glutamic acid with a water molecule occupying a fourth liganding position in a tetrahedral or pentaco-ordination arrangement.

Single HXXXH segments have been observed in several naturally occurring bacterial and vertebrate proteins with demonstrated metal-binding affinities [22]. However, a repeating cluster of such metal-binding sequences has not previously been demonstrated in any other naturally occurring vertebrate proteins. We show here that the expression of this Tx segment is apparently limited to the breast muscle of two orders of birds and suggest a possible link between its metal-binding properties, the phenotype of their breast muscles and the behavioral flight pattern of these avian species.

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2. Experimental

2.1. Troponin T isoform protein preparations

The purified chicken and turkey breast muscle and rabbit skeletal muscle TnTs were prepared as described [12]. The chicken TnT1 to TnT4 isoforms were cloned and expressed in *E. coli* using a pET vector system (J.-P. Jin et al., manuscript in preparation). The four TnT isoform proteins were purified using ion-exchange chromatographies in the presence of 6 M urea and verified by amino acid analysis and N-terminal sequencing.

2.2. Preparation of rabbit antisera against the Tx peptide

The twenty residue Tx peptide conjugated to keyhole limpet hemocyanin (6 mol peptide per mol of carrier) or bovine serum albumin (10 mol peptide per mol of carrier) were purchased from the Alberta Peptide Institute (Edmonton, Canada) and used to prepare antiserum against the Tx peptide. Primary immunization of two male New Zealand White rabbits (2 ~ 2.5 kg) was with 100 μg of the Tx-keyhole limpet hemocyanin conjugate in Freund's complete adjuvant. The animals were boosted three times with 50 μg of the conjugate in Freund's incomplete adjuvant at three-week intervals. An enzyme-linked immunosorbent assay [7] with Tx-bovine albumin conjugate as the immobilized antigen was developed to analyze the rabbit serum specific antibody response.

2.3. Western blotting detection of the expression of Tx structure in different muscles

Total muscle protein extracts from each species were prepared by homogenizing fresh or frozen muscle tissue in 1% SDS at neutral pH. The 12.5% low crosslinker SDS-PAGE and immunoblotting systems were used as described previously [7] except that the electrical transfer to nitrocellulose was carried out in 10 mM 3-[cyclohexylamino]-1-propane-sulfonic acid, pH 11.0, containing 10% methanol. The rabbit antiserum was diluted 1:3000 and [^{125}I]protein G (1 μCi/ml, from ICN Biomedicals, Inc., Costa Mesa, CA) was used as the labelling reagent.

2.4. Metal-affinity chromatography

A mini-column of 2 ml bed volume was prepared with Chelating Fast Flow Sepharose from Pharmacia LKB Technology (Uppsala, Sweden) according to the procedure suggested by the manufacturer. The column was charged with 0.1 M $ZnCl_2$ in 0.5 M NaCl, 50 mM sodium acetate, pH 4.0 and washed with the same buffer without Zn. After equilibration with 0.5 M NaCl, 20 mM sodium phosphate buffer, pH 7.0, containing 2 mM imidazole (buffer A) plus 3 M urea, a mixture of the two recombinant isoforms of TnT1 and TnT3 (1.5 mg of each) was applied to the column in 2 ml of buffer A plus 3 M urea (without imidazole). Following the flowthrough peak, the column was eluted with a linear gradient of imidazole (2 to 100 mM in 49 ml) in the same buffer. Fractions across each of the 280 nm absorbance peaks were examined by 12.5% SDS-PAGE as described previously [7]. Similar affinity chromatographies were performed in the absence or presence of 8 M urea. Chelating Fast Flow Sepharose columns were also charged with $CuSO_4$, $CoCl_2$, $NiCl_2$, $MgCl_2$, $CaCl_2$, $FeSO_4$ and $MnCl_2$ for similar binding experiments as described above for $ZnCl_2$.

2.5. Quantitation of metal contents in chicken muscles

Breast (pectoralis) and leg (gastrocnemius) muscle tissue from adult while leghorn chicken was dissected to remove fat and connective tissues. Muscle pieces (5 mm^3) were dissolved in 0.75 M HNO_3 (prepared in Milli-Q deionized water) at 0.25 g/ml by shaking at 50°C for 24 h. Following centrifugation at 3000 × *g* for 20 min, the supernatant was analyzed for Zn, Cu and Mg on a Leco Plasmarry ICP spectrometer in the Department of Chemistry, University of Alberta.

3. Results and discussion

3.1. The Tx structure in chicken breast muscle TnT

Fig. 1 summarizes the position of the Tx sequence in the N-terminal hypervariable region of chicken skeletal muscle TnT molecule. Secondary structure predictions suggest that the $[HE/AEH]_4$ sequence in the chicken TnT1 isoform may exist in the α-helical form. Structural modelling has shown that for each of the first, second and fourth identical metal-binding sequences (HEEAH), the two histidines and the Glu at the second position can assume a virtually identical orientation as that seen in thermolysin. The third site with the sequence HAEAH and corresponding to residues His-28 to His-32 of Fig. 1 has Glu replaced by Ala at residue 29. It may represent a more weakly binding site. The invariant Glu at position 3 in each of the second, third and fourth repeating pentapeptides may possibly interact through a water molecule occupying a position as a fourth Zn^{2+} ligand in the preceding pentapeptide. These correlations, however, must remain tentative until more detailed structural information becomes available from X-ray crystallographic and/or NMR studies.

3.2. Specific expression of the Tx structure in avian breast muscles

Enzyme-linked immunoabsorbent assay results showed that the rabbit anti-Tx sera obtained were of titers of ~10^{-5} against the chicken breast muscle TnT. Western blots demonstrated the antibody to be highly specific to the TnT isoforms containing the Tx structure (Fig. 2A).

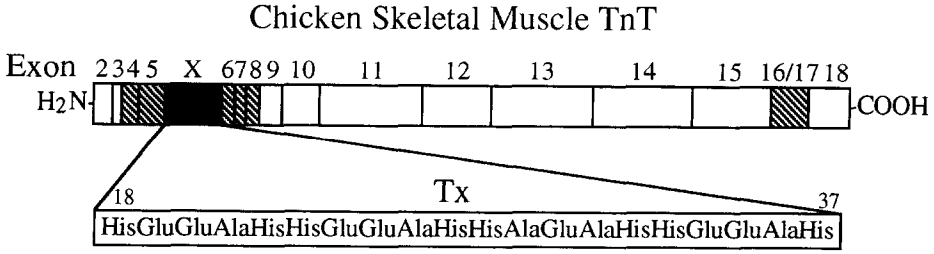

Fig. 1. The metal-binding segment (Tx) found in the TnT1 isoform of chicken breast muscle troponin T. The segment, first identified by protein sequencing [9] and in a cDNA [4], is in a hypervariable and functionally unclear region of the protein encoded by exons 4 to 8 alternatively spliced in a mutually non-exclusive manner. Exons 16 and 17 are alternatively spliced in a mutually exclusive way [2–6]. The 20 residue Tx segment (residues 18–37 in TnT1), largely encoded by exon(s) X and consisting of four repeated HE/AEAH pentapeptides, is predicted to be largely α-helical [34]. Molecular modelling shows that the two His (positions 1 and 5) and the Glu in the second position of each pentapeptide can assume the same orientations as these side chains in the Zn^{2+}-binding site of thermolysin [20].

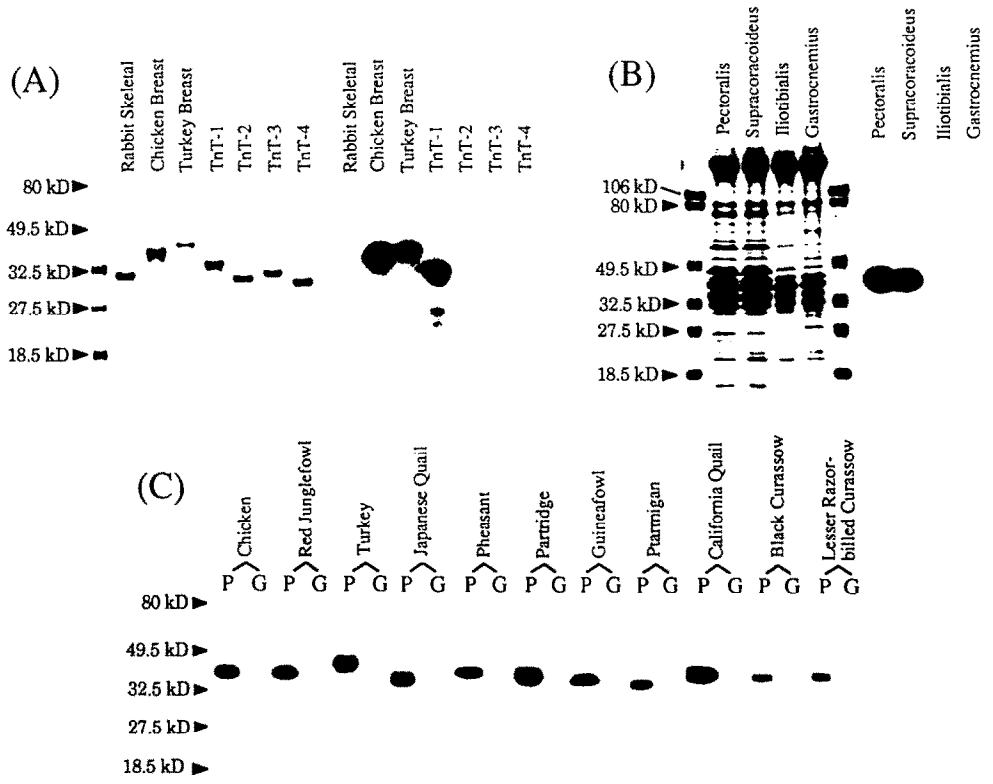

Fig. 2. Detection of the metal-binding Tx segment in various muscles by immunoblotting. (A) Specificity of the anti-Tx serum. The immunoautoradiograph (right) is shown together with the Coomassie blue stained SDS polyacrylamide gel (left). The specific rabbit antiserum recognizes only chicken and turkey breast muscle TnTs and the cloned chicken TnT-1 isoform among the seven TnT proteins examined. TnT-2 to TnT-4 are also isoforms genetically expressed in *E. coli*. Like the rabbit skeletal muscle TnT (11), they do not contain the Tx sequence [4]. (B) Specific expression of the Tx containing TnT isoforms in pectoralis and supracoracoideus of domestic chicken. The left panel is a Coomassie blue-stained SDS-polyacrylamide gel of the total muscle proteins; the right panel is an immunoautoradiograph after blotting on nitrocellulose of an identically prepared gel. (C) Immunoautoradiographs of blots similar to (B) of muscle protein extracts from different birds in the orders *Galliformes* and *Craciformes*. The results show that the Tx epitope was detected only in their breast (P, pectoralis) muscle TnTs. All samples from avian leg muscles (G, gastrocnemius pars interna), heart muscles, mammalian skeletal muscles and breast muscles from species in other orders of birds gave negative results (see also Table 1). At least three Tx-containing TnT variants were detected in young (~8 weeks old) developing chicken pectoralis (results not shown).

The results from Western blotting examination of the tissue and species distribution of this unique structure (Fig. 2 and Table 1) demonstrate that the Tx epitope is present in the breast but not leg muscles of the nine *Galliformes* species examined. These include seven specimens from the *Plasianidae* family and one each from the *Numididae* and *Odontophoridae*. A weaker reaction was also observed for breast muscle TnTs in two species of the order *Craciformes*. No Tx epitope was detected in the breast or leg muscles of any other avian species. These included 17 specimens from 15 families belonging to 10 different orders of birds. Tx epitope was also absent in the skeletal muscles of rat and rabbit, the cardiac muscles of chicken, rabbit and rat and in the cloned chicken skeletal muscle isoforms TnT2, TnT3 and TnT4 which do not contain the Tx sequence [4]. A homology search of the data banks revealed that the chicken Tx sequence showed 64–73% nucleotide sequence and 54% amino acid sequence identity with portions of the histidine/alanine-rich proteins present in *Plasmodium falciparum* and *Plasmodium lophurae*, the latter being an avian malarial parasite [23–25]. These observations and the fact that the epitope is absent (see Table 1) in the sister orders *Anseriformes* (duck, goose, teal), *Struthioniformes* (ostrich) and *Tinamiformes* (tinamou) suggest that the trait might have been acquired by an early recombinational event involving the genomes of the avian malarial parasite and an early ancestor of the *Galliformes* and *Craciformes* following their divergence from other bird orders.

3.3. Specific binding of Tx containing TnTs to metal-affinity columns

A chromatographic profile of a mixture of the recombinant TnT isoforms TnT1 and TnT3 applied to a metal-affinity column charged with Zn^{2+} is shown in Fig. 3. The results clearly show that T1 is retained by the column and is only eluted with an imidazole gradient. Isoform TnT3, devoid of the Tx sequence, is not retained by the column and appears in the flow-through. In further experiments, purified or partially purified TnT preparations from several sources were applied singly or as a mixture to the Zn-charged column in buffer A containing

3 M urea as in Fig. 3. Rabbit skeletal muscle TnT and recombinant chicken TnT2 and TnT4 which do not contain the Tx sequence were not retained by the column. Chicken, Japanese quail and turkey breast muscle TnTs, all containing the Tx epitope, were only eluted after application of a linear imidazole gradient in the region of 30–50 mM.

In other experiments, columns were prepared and charged with a variety of metals as described in section 2. Following equilibration with buffer A (without urea), purified chicken breast muscle TnT (2 mg) was applied to each column followed by step-wise increases in imidazole concentration of the eluting buffer A (without urea). The imidazole concentration required for elution from each metal-affinity column was: Cu^{2+}, >200 mM; Ni^{2+}, 130 mM; Zn^{2+}, 40 mM; Co^{2+}, 40 mM. The chicken breast muscle TnT was not retained on the Mg^{2+}, Ca^{2+}, Mn^{2+} or Fe^{2+} columns.

It has also been shown that this cluster of four metal-binding sites provides a rapid procedure for the purification of the recombinant TnT1 isoform on a Zn^{2+} affinity column in 3 M urea. For the purification of TnT isoforms containing the Tx sequence in chicken, turkey and quail breast muscle, Ni^{2+} or Cu^{2+} charged columns can be used in the presence of 8 M urea and/or 1 M salt for dissociation from other myofibrillar proteins. Binding of the Tx sequence is considerably stronger than that reported for a single HXXXH site [26,27] suggesting that the engineering of a cluster of such sites in a recombinant protein should permit greater versatility in the choice of chromatographic conditions for purification purposes.

3.4. *Metal contents of breast and leg muscles of avian species*

The observation that the metal-binding Tx sequence was restricted to the breast muscles of the two bird orders *Galliformes* and *Craciformes* prompted us to determine the metal contents of some of these avian muscle tissues. Preliminary data on the total Zn levels of Tx containing breast muscles (4 *Galliformes* species) compared with Tx minus breast muscles (3 species) indicate a significantly lower total Zn concentration in the former

Table 1. Expression of the Tx Epitope in Avian Muscles

Order	Family	Species: Latin Name	Species: English Name	Tx Epitope: Pectorals	Tx Epitope: Leg Muscles
Galliformes	Phasianidae	*Gallus domesticus*	Domestic Chicken	+	−
		Gallus gallus	Red Junglefowl	+	−
		Coturnix japonica	Japanese Quail	+	−
		Perdix perdix	Grey Partridge	+	−
		Phasianus colchicus	Ring-necked Pheasant	+	−
		Meleagris gallopavo	Domestic Turkey	+	−
		Lagopus lagopus	Willow Ptarmigan (Grouse)	+	−
	Numididae	*Numida meleagris*	Guineafowl	+	−
	Odontophoridae	*Lophortyx californica*	California Quail	+	−
Craciformes	Cracidae	*Crax tomentosa*	Lesser Razor-billed Curassow	+	−
		Crax alector	Black Curassow	+	−
Anseriformes	Anatidae	*Anas platyrhynchos*	Pekin Duck	−	−
		Anas discors	Blue-winged Teal	−	−
		Anser anser	Domestic Goose	−	−
Tinamiformes	Tinamidae	*Eudromia elegans*	Elegant Crested-Tinamou	−	−
Struthioniformes	Struthionidae	*Struthio camelus*	Ostrich	−	−
Piciformes	Picidae	*Sphyrapicus varius*	Yellow-bellied Sapsucker	−	−
Trochiliformes	Trochilidae	*Selasphorus rufus*	Rufous Hummingbird	−	−
Strigiformes	Strigidae	*Nyctea scandiaca*	Snowy Owl	−	−
Columbiformes	Columbidae	*Columba livia*	Domestic Pigeon	−	−
Gruiformes	Rallidae	*Fulica americana*	American Coot	−	−
Ciconiiformes	Laridae	*Larus delawarensis*	Ring-billed Gull	−	−
	Accipitridae	*Circus cyaneus*	Northern Harrier	−	−
	Podicipedidae	*Podiceps grisegena*	Red-necked Grebe	−	−
	Ardeidae	*Ardea herodias*	Great Blue Heron	−	−
	Gaviidae	*Gavia immer*	Common Loon	−	−
Passeriformes	Bombycillidae	*Bombycilla cedrorum*	Cedar Waxwing	−	−
	Corvidae	*Pica pica*	Common Magpie	−	−

The results of Western blot analysis of the breast and leg muscles of 28 avian species from 19 families and 12 orders are summarized. The classification and evolutionary relationships of these birds are as described by Sibley and Ahlquist [33]. +, Tx epitope detected; −, no Tx epitope detected. The results show that the Tx epitope is present only in the breast muscles of all three families of *Galliformes* and at least two species of the *Craciformes*.

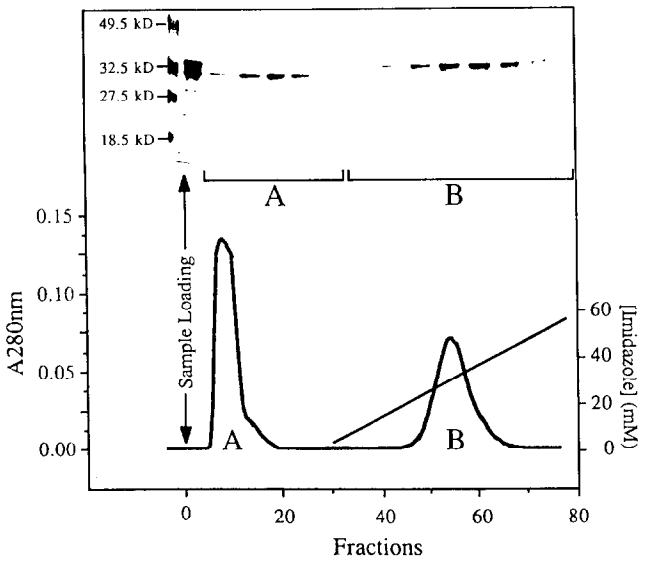

Fig. 3. Zn-affinity chromatographic analysis of the specific metal-binding of Tx sequence. A mixture of partially purified cloned chicken TnT1 and TnT3 isoforms was chromatographed on a Zn-affinity column and the $A_{280\,nm}$ peak fractions were analyzed by SDS-PAGE. The results show that TnT1 was specifically retained on the column while TnT3 and contaminant proteins passed through. The difference in primary structure between these two isoforms is only in the N-terminal hypervariable region in which TnT1 contains the Tx segment encoded by exon X while TnT3 contains two different segments encoded by exons 4 and 7 (see [4]). The calculated molecular weights and isoelectric points of TnT1 and TnT3 are 32,340, 6.64; 31,142 and 6.49, respectively.

group ($P < 0.01$). More extensive total Zn and Cu analyses [28] on the breast and leg muscles of fifteen white leghorn chickens have also been carried out. In the breast muscle, the Zn and Cu concentrations were 0.26 ± 0.03 and 0.024 ± 0.002 mmol/kg of dry weight, respectively. Corresponding values in the leg muscles were 0.59 ± 0.10 and 0.042 ± 0.004 mmol/kg. These differences in Zn^{2+} and Ca^{2+} levels for breast and leg muscles are highly significant ($P < 0.001$). On the other hand, the total Mg concentrations of chicken breast and leg muscles were very similar (52.3 ± 2.58 and 50.8 mmol/kg, respectively). These Zn and Cu levels are of the same order of magnitude as the concentration in breast muscle of the Tx metal binding sites of TnT, a major structural protein in the cell. Assuming that the myofibrillar proteins make up ~70% of the total dry weight of the muscle mass and that whole troponin represents 5% of the protein content of the myofibril [29], it can be calculated that the concentration of Tx metal-binding sites would be ~1–2 mmol/ kg of dry muscle. These figures suggest that free metal ion concentrations could be significantly affected by the expression of the Tx containing isoform(s) in these breast muscle cells.

While an interpretation of the differences between chicken breast and leg muscle total Zn and Cu levels reported above must be tempered with caution (i.e. differences in vasularization, mitochondrial content, etc.), the lower values seen in chicken breast tissue indicate a specialized adaptation towards this end in these two orders of birds. The present demonstration of the Tx metal-binding cluster in these same tissues would suggest that the free metal concentration would be further reduced by its presence.

The *Galliformes* order of birds is characterized by a high ratio of breast muscle to total body muscle mass [30], by a high content of fast glycolytic fiber type in the pectoralis muscle [31] and by an explosive but short-lived flight pattern. In light of the established roles [22,32] of metal ions in metabolism and in gene transcription, it would seem more than coincidental that the expression of this metal-binding cluster in a major muscle protein is limited to the pectoralis muscles of birds with these characteristics. We suggest that the acquisition of this particular genetic trait may have been an important event, among others, in the evolution of the *Galliformes* and *Craciformes* orders of birds.

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