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Identification of catalytically important amino acids in human ceruloplasmin by site-directed mutagenesis

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Abstract The involvement of amino acid residues previously proposed on the basis of structural data to have roles in the ferroxidase and diamine oxidase activities of human ceruloplasmin was investigated. Variants of human ceruloplasmin, in which residues proposed to be involved in electron transfer and/or iron-binding had been altered by site-directed mutagenesis, were expressed in HEK293 cells. E633A and E597A/H602A variants exhibited reduction in both activities by 50–60% compared to recombinant wild-type ceruloplasmin. The variant E935A/H940A had reduced ferroxidase activity (50%) but unaltered diamine oxidase activity, whereas the variant E971A exhibited enhanced diamine oxidase activity. For the L329M variant, both activities were identical to those of wild-type ceruloplasmin.

Key words: Ceruloplasmin; Copper; Ferroxidase; Iron; Diamine oxidase; Site-directed mutagenesis

1. Introduction

Ceruloplasmin (CP) is a member of the blue multi-copper oxidase family of enzymes that includes laccase, ascorbate oxidase, Fet3, bilirubin oxidase and hephaestin. The primary structure of CP suggests that it also shares homologous domains with blood coagulation factors V and VIII [1]. CP is synthesised primarily in the liver and secreted into the blood as a single-chain glycoprotein containing six prosthetic copper atoms. In humans, circulating CP comprises 1046 amino acids, exhibits an apparent molecular mass of ~132 kDa and has a normal plasma concentration of ~0.3 mg/ml. Greater than 95% of circulating copper is bound to CP; in addition to its prosthetic copper atoms, CP loosely binds several other copper ions [2].

CP can oxidise several substrates, including Fe^{2+}, aromatic amines and phenols. CP is efficient at oxidising Fe^{2+} in vitro without generating free radicals: four one-electron oxidations of Fe^{2+} are coupled to the four-electron reduction of O_2 to $2H_2O$ [3]. Although a potential role in oxidising biogenic amines such as adrenaline has been proposed [4], several observations including a low K_m for oxidation of Fe^{2+} (0.6 μM [5]) indicate that iron may be the most important physiological substrate of CP. CP can load iron into transferrin and ferritin in vitro, although whether this occurs physiologically is unknown.

Studies of individuals with aceruloplasminaemia, a fatal disease characterised by an absence of plasma CP, have provided clear evidence that CP is involved in iron homeostasis [6]. Investigations utilising animals, cultured cells, knockout mice and yeast (e.g. [7–11]) have also highlighted an important role for the ferroxidase activity of CP, though a precise description of its functions in iron homeostasis has yet to be resolved.

Determination of the three-dimensional structure of human CP (hCP) [12] has furnished clues to its oxidase function. The enzyme contains six β-barrel domains arranged in a triangular array. Three of the six prosthetic copper atoms occupy mononuclear type 1 sites in domains 2, 4 and 6 whereas the remaining three form a trinuclear cluster at the interface of domains 1 and 6. The trinuclear cluster comprises a pair of coupled type 3 copper atoms linked to a type 2 site. The arrangement of the trinuclear cluster and domain 6 mononuclear site is similar to that observed in ascorbate oxidase, laccase and Fet3 [13,14]. The mononuclear sites are thought to be involved in intramolecular electron transfer as the initial acceptors of reducing equivalents from the substrate, whereas binding and reduction of O_2 occurs at the trinuclear cluster [3,15].

Studies of metal ion-soaked crystals have identified two putative cation-binding sites in a negatively charged pocket near to the copper atoms in domains 4 and 6 [16]. Binding of Fe^{2+} at these sites has been proposed as the first step in the ferroxidase catalytic mechanism, followed by oxidation of the metal and translocation of the resultant Fe^{3+} [16]. Soaking experiments with *p*-phenylenediamine suggest that aromatic diamines bind at the bottom of domain 4, remote from the mononuclear copper atom in this domain [17].

Analysis of the three-dimensional structure of hCP also indicated potential electron transfer pathways between the prosthetic copper atoms [15,16]. Here we report the effects on two of the enzymatic activities of hCP caused by the substitution of residues postulated, on the basis of structural data, to be involved in intramolecular electron transfer and/or iron-binding. As far as we are aware, this report represents the first functional study of the importance of these residues for the catalytic mechanism of ceruloplasmin.

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Abbreviations: CP, ceruloplasmin; ELISA, enzyme-linked immunosorbent assay; hCP, human ceruloplasmin; rhCP, recombinant human ceruloplasmin; TBS, Tris-buffered saline

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2. Materials and methods

2.1. Construction of hCP expression vector and site-directed mutagenesis

A cDNA encoding hCP was subcloned from pBK-CMV-CP into the plasmid pCI-neo (Promega, Madison, WI, USA) to produce the vector pCI-neo-CP. Mutagenesis was performed using pCI-neo-CP as the template and the oligonucleotides listed in Table 1. Vector (0.03 pmol) and oligonucleotide (25 pmol) were annealed, T4 DNA polymerase, T4 ligase, dNTPs and ATP were added, and extension and ligation were effected by incubating the mixture at 37°C for 2 h. After treatment with *Dpn*I (0.1 U for 30 min at 37°C), the reaction was used to transform XL*mutS* (Kan^S) *Escherichia coli* cells (Stratagene, La Jolla, CA, USA). Transformants were selected with ampicillin, and appropriate colonies were identified by PCR and restriction endonuclease analysis (see Table 1 for restriction sites used as markers). All mutations were confirmed by DNA sequence analysis of the vectors. Enzymes were purchased from New England Biolabs (Beverly, MA, USA).

2.2. Cell transfection and culture

A human embryonic kidney (HEK293) cell line was transfected with each vector using DOTAP Liposomal Reagent (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Cells were cultured in a 5% CO_2 atmosphere at 37°C in Dulbecco's modified Eagle's medium/Nutrient Mix F12 (DMEM/F12; Life Technologies, Rockville, MD, USA) containing 5% (v/v) newborn calf serum (Life Technologies), and clonal cell lines were isolated by selection with 0.75 mg/ml Geneticin (Life Technologies). For expression of hCP, cells were grown to ~70% confluence, the serum-containing medium was removed, and the cells were washed three times with DMEM/F12 without phenol red. The medium was replaced with DMEM/F12 without phenol red containing 0.5% (v/v) KnockOut SR serum-free supplement (Life Technologies) and 10 μM $CuSO_4$. Conditioned medium was harvested after 2–5 days, dialysed against Chelex 100-treated H_2O, concentrated, and frozen at −80°C.

2.3. Western blot analysis

Protein samples (denatured/reduced) were resolved in SDS/7.5% (w/v) polyacrylamide gels and electroblotted to nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was blocked with 10% (w/v) skim milk powder in Tris-buffered saline (TBS; 20 mM Tris–HCl, 154 mM NaCl, pH 7.0), then incubated with goat anti-hCP antiserum (Sigma, St. Louis, MO, USA) followed by washing and incubation with horseradish peroxidase-conjugated rabbit anti-goat IgG (Sigma). Blots were developed with an ECL Western Blotting Kit (Amersham Pharmacia Biotech).

2.4. Quantification of hCP

The concentration of recombinant hCP (rhCP) was determined by competition ELISA. Purified hCP (Calbiochem, La Jolla, CA, USA) was coated in 96-well microtitre plates (10 μg/ml in TBS, 50 μl per well) overnight at 4°C, then the wells were blocked for 1 h with 0.5% (w/v) bovine serum albumin (Sigma) in TBS. Samples were pre-incubated for 1 h with rabbit anti-hCP IgG (DAKO, Glostrup, Denmark), then added to the blocked wells and incubated overnight at 4°C. After washing, alkaline phosphatase-conjugated mouse monoclonal anti-rabbit IgG (Sigma) was added, and the plates were incubated for 45 min at room temperature. The wells were washed, and *p*-nitrophenyl phosphate liquid substrate (Sigma) was added. The reactions were quenched with 3 M NaOH, and the A_{405} was measured in a microtitre plate reader (Dynex Technologies, Ashford, Middlesex, UK). The concentration of rhCP was determined from a standard curve generated with purified hCP (Calbiochem). The limit of detection of the assay (3 S.D. above background) was 2.5 ng/ml, and the assay was linear from 80 to 1200 ng/ml. All assays were performed in triplicate.

2.5. Enzyme assays

Diamine oxidase activity was measured with the substrate *O*-dianisidine dihydrochloride (Sigma) [18,19]. Assays were performed at 37°C with 1 μg CP in 60 mM sodium acetate buffer, pH 5.0 containing 1.6 mM *O*-dianisidine. One unit of activity (U) is defined as 1 μmol of *O*-dianisidine oxidised per min, based on a molar extinction coefficient, $\varepsilon_{540} = 9.6 \times 10^3\ M^{-1}\ cm^{-1}$. Ferroxidase activity was measured using a discontinuous assay in which oxidation of Fe^{2+} is monitored by quantifying the Fe^{2+} remaining in the solution after a set period of time [20]. Assays were performed at 37°C with 1 μg CP in 0.42 M sodium acetate buffer, pH 5.8 containing 24.5 μM $Fe(NH_4)_2(SO_4)_2$ (J.T. Baker Chemical, Phillipsburg, NJ, USA). Samples of the reactions were removed at 10 min and 30 min and quenched with ferrozine (Sigma). The concentration of Fe^{2+} was determined from the absorbance of the ferrozine–Fe^{2+} complex ($\varepsilon_{562} = 2.79 \times 10^4\ M^{-1}\ cm^{-1}$). As a negative control, CP was replaced by EDTA and, as a positive control, it was replaced with Chelex 100-treated H_2O. One unit of activity (U) is defined as 1 μmol of Fe^{2+} oxidised per min.

3. Results and discussion

We assessed the effect of various amino acid substitutions upon the ferroxidase and diamine oxidase activities of hCP. We replaced residues with alanine that have been postulated, on the basis of structural analyses [12,16], to be potentially involved in electron transfer and/or the labile cation-binding sites of the protein. To our knowledge, this work represents the first functional study of the importance of these residues for the catalytic mechanism of CP. One of the residues of the domain 2 mononuclear copper site (L329) was also replaced by methionine.

Vectors encoding each of the mutated proteins and wild-type hCP were used to stably transfect HEK293 cells. As the presence of an intron has been shown to be requisite for efficient expression of CP in mammalian cells [21], we employed a vector containing a chimaeric intron upstream of the cloning site. Cells transfected with the pCI-neo-CP vector secreted wild-type rhCP, whereas mock-transfected cells did not produce detectable amounts of the protein (Fig. 1). Likewise, each of the five mutated hCP proteins was also synthesised and secreted (Fig. 1). In all cases, only a single band with an apparent mass comparable to plasma-derived hCP (~130 kDa) was observed on Western blots, indicating that the secreted proteins were intact and undegraded. CP concentrations in the conditioned medium were up to 2.5 μg/ml as determined by ELISA. Wild-type rhCP was functionally active and able to oxidise both Fe^{2+} and *O*-dianisidine at rates comparable to plasma-derived hCP (Fig. 2). Expression of the ferroxidase and diamine oxidase activities was enhanced by inclusion of $CuSO_4$ in the medium, and both activities were

Table 1
Oligonucleotides used for site-directed mutagenesis of hCP

Mutation	Oligonucleotide sequence[1]
L329M	5′ ggaaaaaggcttg**cat**gccggctttcagatgg 3′ *Sph I*
E597A/H602A	5′ GAAGACTTTCAG**GCC**TCTAATAAAATG**GCG**TCCATGAATG 3′ *Stu I* *Bsa HI*
E633A	5′ gtacatcggc**cgc**atttccggcg 3′ *Eag I*
E935A/H940A	5′ GGAATTCATA**GCT**AGCAATAAAATG**GCT**GCTATTAATGG 3′ *Nhe I* *~~Sph I~~*
E971A	5′ cagtgtgtaagtcaat**tgc**attgcccattccc 3′ *Mfe I*

Oligonucleotides shown in upper case anneal to the coding (+) strand, those in lower case to the non-coding (−) strand. Codons specifying the mutated amino acids are shown in bold. Restriction endonuclease sites incorporated into the oligonucleotides as markers are underlined. The position of a unique *Sph*I site that was knocked out by the E935A/H940A oligonucleotide is also indicated.

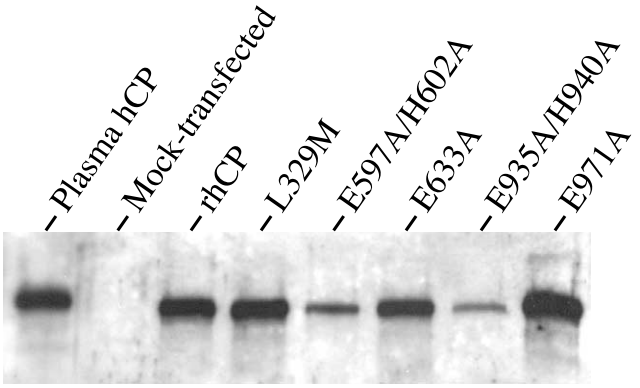

Fig. 1. Expression of hCP by transfected HEK293 cells. HEK293 cells were stably transfected with either pCI-neo (mock transfection), pCI-neo-CP (for expression of wild-type CP (rhCP)) or pCI-neo-CP vectors in which the hCP cDNA had been altered by site-directed mutagenesis to encode the denoted single or double amino acid substitutions. Conditioned medium (15 μl) from clonal cell-lines was denatured and reduced, resolved by SDS–PAGE, and Western blotted with antibodies against hCP. Bands were visualised by a chemiluminescence-based method. The left-most lane contained plasma-derived hCP (10 ng).

minimal in medium from cells cultured without additional copper (data not shown).

The substitution E633A reduced both the ferroxidase and diamine oxidase activities of hCP by ~60% (Fig. 2), indicating that E633 may be important in the general catalytic mechanism of the enzyme. This finding is consistent with the role proposed for this residue in a potential electron transfer pathway linking the mononuclear copper sites in domains 2 and 4 [16] (Fig. 3). However, the possible importance of the pathway (Cu2-H324-(H-bond)-E633A-A634-D635-V636-H637-Cu4) has been questioned because of the relatively long (3.7 Å) distance between the N^{ε} atom of H324 and the side chain O^{δ} atom of E633 [16]. Notably, the moderate resolution of the structure determined by X-ray crystallography (~3.0 Å) lends some uncertainty to the length of the hydrogen bond. Additionally, Farver et al. [15] have suggested that the mononuclear copper atoms in domains 2 and 4 may play little, if any, role in catalysis and that electron transfer is not expected to occur between any of the three mononuclear sites. This view is not supported by our observations nor those of other studies: analysis of sheep CP partially depleted of its type 1 copper atoms suggests that all three mononuclear sites act as a pool and may be equally active in electron transfer [4,22]. Furthermore, destruction of the mononuclear site in domain 6 of hCP does not completely abolish diamine oxidase activity, indicating that another type 1 site is able to contribute to the catalytic mechanism [23]. The three mononuclear sites are in sufficient proximity to permit electron transfer (within ~18 Å). Our results suggest that the proposed pathway may participate in shuttling electrons released during the oxidation of both Fe^{2+} and diamines because the ferroxidase and diamine oxidase activities of hCP were affected to a similar degree by the E633A substitution. As noted below, however, the mononuclear copper in domain 2 of hCP has been shown recently to have a very high potential that argues against a role for this site in the human protein, though it is possible that it might serve a function as a reservoir of electrons.

X-ray diffraction studies of hCP have identified separate binding sites for Fe^{2+} and diamines. The diamine-binding site is located near W669 in domain 4. Consequently, the oxidation of diamine substrates may be achieved as electrons are channeled through the domain 4 mononuclear copper centre [17]. Crystal-soaking experiments indicate that Fe^{2+} binds at two labile cation-binding sites in domains 4 and 6 [16]. Binding appears to occur through the displacement of non-prosthetic copper atoms followed by release of an electron and translocation of Fe^{3+} to nearby holding sites. The ligands of the domain 6 labile cation-binding site are E272, E935, H940 and D1025 (Fig. 3), of which E935 has also been implicated in Fe^{3+} translocation [16,17]. This binding site, which is located near that proposed to bind biogenic amines [17], is close to the mononuclear copper centre in domain 6, and electrons could easily be transferred to the copper atom through its H1026 ligand: subsequent transfer to the trinuclear centre is most likely achieved via a Cys-His pathway similar to that observed in ascorbate oxidase [24].

Substitutions of two of the amino acids comprising the proposed domain 6 cation-binding site of hCP, E935 and H940, was accompanied by an ~50% decrease in ferroxidase activity, whereas diamine oxidase activity was unaffected (Fig. 2). This observation is consistent with the notion that one of the two iron-binding sites on the molecule should be disrupted by this substitution and provides functional evidence that the domain 6 site is important for the ferroxidase mechanism of hCP. Likewise, substitutions of E597 and H602, two of the ligands proposed to constitute the labile cation-binding site in domain 4 – the others are D684 and E971 (Fig. 3) – caused an ~50% decrease in the ferroxidase activity of hCP (Fig. 2). However, in this case the diamine oxidase activity was also

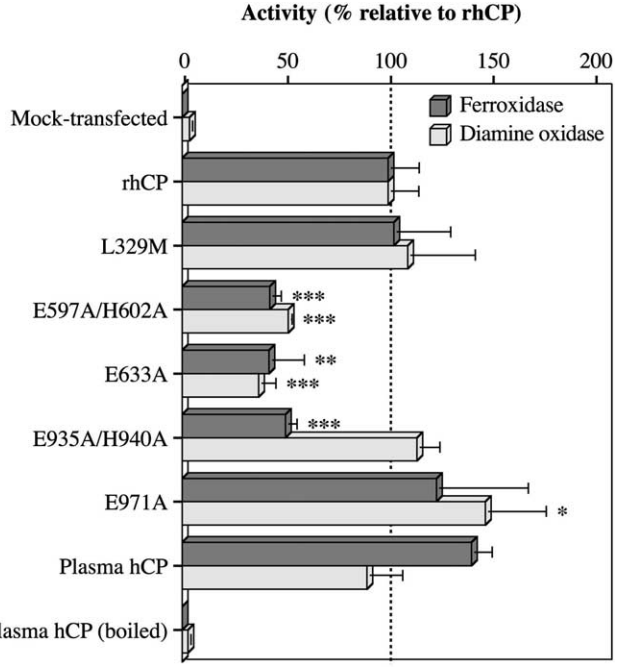

Fig. 2. Activities of recombinant wild-type and mutated hCP. The ferroxidase and diamine oxidase activities of the recombinant proteins were measured and are plotted relative to wild-type CP (rhCP). The value 100% corresponds to an activity of 0.154 U/mg for oxidation of Fe^{2+} and 0.114 U/mg for oxidation of *O*-dianisidine. Data are the average of four measurements (duplicates for preparations from two separate clonal cell lines), and error bars represent +1 S.D. Asterisks denote a significant difference from rhCP: $*P < 0.01$; $**P < 0.005$; $***P < 0.0005$.

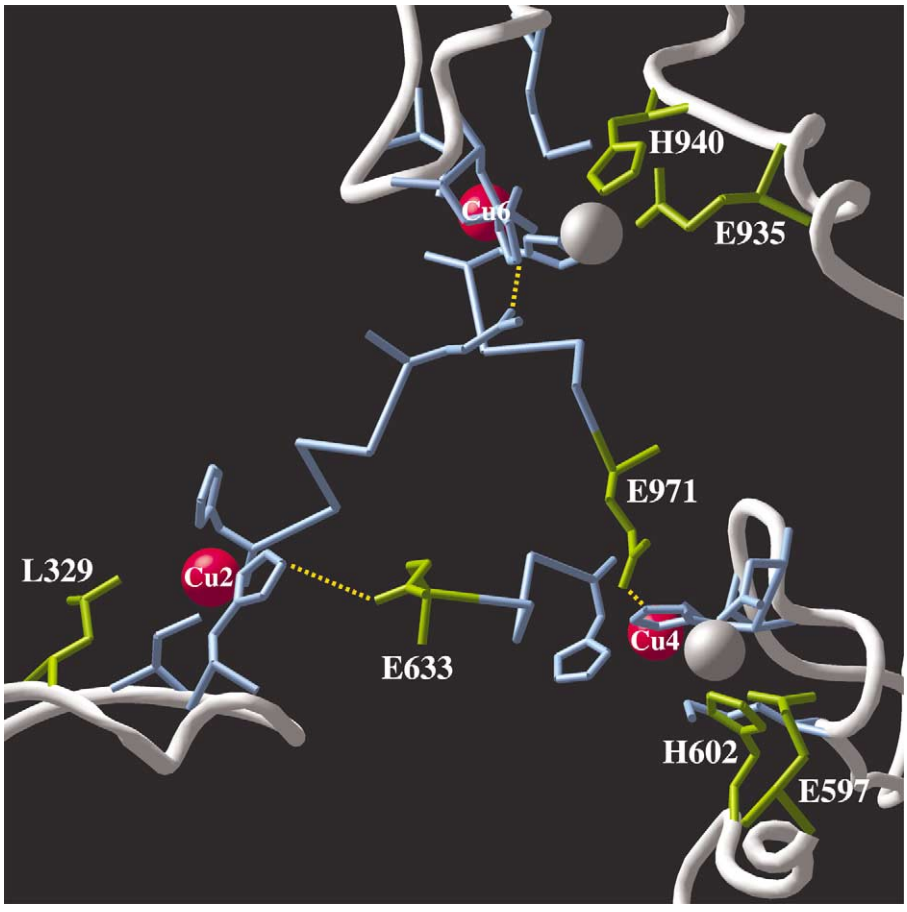

Fig. 3. Structural locations of residues mutated in hCP. Model shows the labile cation-binding sites in domains 4 and 6 of hCP and the proposed electron transfer pathways linking the three mononuclear copper sites. The prosthetic copper atoms are depicted as red spheres and metal atoms bound at the labile sites as grey spheres. The side chains of the residues that were mutated to Ala in this study are coloured light green and are labelled. The model was generated from the published coordinates for hCP [12,16], using the program Swiss-PdbViewer (available online at www.expasy.ch/spdbv/mainpage.html).

reduced to a comparable degree. It is thus possible that the local loss of charged residues might have perturbed the diamine-binding site or perhaps even the environment of the nearby mononuclear copper centre in domain 4.

Substitution of E971 had no detrimental effect on ferroxidase activity and somewhat enhanced the diamine oxidase activity (Fig. 2). This unanticipated result argues against the proposal that E971 has roles both in electron transfer and as a ligand of the labile cation-binding site in domain 4 [16] (Fig. 3). The proposed pathway in this case follows the route Cu4-H685-(H-bond)-E971-I972-D973-L974-H975-Cu6, thus linking the mononuclear copper sites in domains 4 and 6, both of which are active redox centres [3]. It therefore appears that E971 is not a requisite ligand of the iron-binding site in domain 4 and that an alternative pathway may exist for shuttling electrons from the domain 4 to domain 6 mononuclear copper centre, possibly via the copper centre in domain 2.

We also tested the effect of substituting L329, a non-coordinating residue in the domain 2 type 1 copper centre (Fig. 3), with Met. The presence of Leu at this position is unusual in that the site is typically occupied by an axial Met residue in type 1 copper centres. Other multi-copper oxidases that contain Leu at the corresponding position are Fet3 and certain laccase isozymes [3]. In these enzymes, as in hCP, the copper atom is bound by the side chains of two His residues and a Cys residue. These tri-coordinate sites have a relatively high reduction potential (possibly exceeding 1 V), and it has been argued that in hCP the copper atom remains in a permanently reduced and, hence, redox-inactive state [3,25]. No effect on either ferroxidase or diamine oxidase activity was observed for the L329M substitution (Fig. 2), an alteration that might be expected to decrease the reduction potential of the copper centre. This observation concurs with a recent study of hCP expressed in yeast, which reported that the L329M substitution did not alter either the ferroxidase activity or spectroscopic properties of the molecule [23]. Presumably, this substitution is necessary but not sufficient to restore functional activity to this copper-binding site.

In conclusion, using site-directed mutagenesis and analysis of rhCP expressed in a human cell line, we have investigated the functional importance of certain residues that have been proposed, based on structural data, to play a key role in the ferroxidase and diamine oxidase functions of this protein.

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