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The dimer contact area of sorghum NADP-malate dehydrogenase: role of aspartate 101 in dimer stability and catalytic activity

Isabelle Schepens[a], Paulette Decottignies[a], Eric Ruelland[a], Kenth Johansson[b], Myroslawa Miginiac-Maslow[a,*]

[a] *Institut de Biotechnologie des Plantes, UMR 8618 CNRS, Bâtiment 630, Université de Paris-Sud, 91405 Orsay Cedex, France*
[b] *Department of Molecular Biology, Swedish University of Agricultural Sciences Biomedical Center, P.O. Box 590, S-751 24 Uppsala, Sweden*

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Abstract During thioredoxin-mediated activation of chloroplastic NADP-malate dehydrogenase, a homodimeric enzyme, the interaction between subunits is known to be loosened but maintained. A modeling of the 3D structure of the protein identified Asp-101 as being potentially involved in the association between subunits through an electrostatic interaction. Indeed, upon site-directed substitution of Asp-101 by an asparagine, the mutated enzyme behaved mainly as a monomer. The mutation strongly affected the catalytical efficiency of the enzyme. The now available 3D structure of the enzyme shows that Asp-101 is protruding at the dimer interface, interacting with Arg-268 of the neighbouring subunit.

Key words: Dimer; Monomer; NADP-malate dehydrogenase; Site-directed mutagenesis; Thioredoxin

1. Introduction

Among all the chloroplast enzymes reversibly activated by light via a thiol/disulfide interchange with reduced thioredoxin [1] NADP-malate dehydrogenase (NADP-MDH: EC 1.1.1.82) is certainly the one for which a wealth of biochemical data is available concerning the activation mechanism. Recent site directed mutagenesis [2] and structural [3,4] studies reached convergent conclusions concerning the auto-inhibition of the oxidised form of the enzyme by its C-terminal extension which loops back into the active site, thus obstructing substrate access. Indeed, compared to the permanently active, NAD-dependent malate dehydrogenases, the light-activated form has a longer amino acid sequence and features two extensions, one at the C-terminus, the other at the N-terminus, each of them bearing a thioredoxin-reducible disulfide bridge [3–7]. The interpretation of the role of the N-terminal bridge is less straightforward. The portion of the N-terminal extension containing the disulfide bridge forms a short helix inserted between the two subunits of this homodimeric enzyme and makes a number of hydrophobic interactions with both subunits, thus rigidifying the dimeric structure. This feature has been proposed to prevent the active site from reaching the flexibility necessary for catalytic activity. The N-terminal extension is thus believed to be involved in dimerisation, an assumption supported by the observation that the deletion of 33 amino acid residues at the N-terminus (ΔN mutant) results in a monomeric protein [8–10]. The reduction of the N-terminal disulfide loosens the interaction between subunits, which can be separated more easily and this should increase the flexibility of the 3D structure of pig cytosolic MDH [5] led us to suggest that the interaction between subunits could also involve an interaction between Arg-204, one of the two arginines of the active site known to be implicated in oxaloacetate binding in the pig enzyme, and Asp-101 from the neighbouring subunit. These two residues were expected to be linked by a salt bridge. The modification of the interaction between subunits could disrupt this bridge and help the active site to reach a fully active conformation. In yeast mitochondrial NAD-MDH [12] the position corresponding to Asp-101 is occupied by a Histidine. This His has been demonstrated to play a role in the stability of the dimer and also in the catalytic activity of the enzyme. These observations prompted us to investigate the role of Asp-101 in the dimerisation and catalytic activity of NADP-MDH by substituting this residue by an asparagine in order to eliminate a possible electrostatic interaction with Arg-204 of the neighbouring subunit. The results are in favour of a role for this residue in dimer stability and catalytic activity.

2. Materials and methods

2.1. Materials

Pfu DNA polymerase, oligonucleotides, restriction endonucleases, T4 DNA ligase, were obtained from Promega, Appligene and Life Technologies. DEAE-Sephacel and Matrex red A chromatographic supports were respectively from Sigma and Millipore. The monoQ HR5/5 HPLC column and the radiolabels were from Amersham-Pharmacia Biotech. Anti-rabbit IgG coupled to peroxidase and biochemical reagents were obtained from Sigma-Aldrich.

The pUC9 and pET*mdh* vectors, described in [8] were used in the mutagenesis strategy. *Escherichia coli* DH5α F′ (Life Technologies) was used to produce high yields of pET and pUC vectors. *E. coli* BL21 (DE3) strain [hsdS gal (λc Its 857 ind1 Sam7 nin lacUV5-T7 gene 1)] was used for the production of wild type (WT) and mutated NADP-MDHs encoded by recombinant pET vectors [13]. Bacteria were grown, at 37°C on Luria broth medium, supplemented with ampicillin 50 μM when the bacteria carried pET or pUC plasmids.

2.2. Site directed mutagenesis of the NADP-MDH cDNA and DNA sequencing

Site-directed mutagenesis was performed by PCR using the pET*mdh* vector as a template. The following oligonucleotides were used: D101N-up: GCTATGGAACTGGAG**AAT**TCGCTATATCCATTGCTG; D101N-rev: CAGCAATGGATATAGCGA**ATT**CTCCAGTTCCATAGC; *Nhe*I-rev: CCTGCTTTGAGTGCTAGCTGGCACTTTGCTC; Near-up: CACAACGGTTTCCCTCTAGAAATAATTTTG.

*Corresponding author. Fax: (33)-1-69 33 64 23.
E-mail: miginiac@ibp.u-psud.fr

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Mutagenic bases are in bold. The Near-up oligonucleotide hybridises to the pET plasmid 50 bp from the 5′ end of the MDH cDNA. Three PCR steps were necessary to obtain the mutated fragment. The first PCR reaction was run with the D101N-rev and the Near-up oligonucleotides and the second PCR reaction with the D101N and the *Nhe*I-rev oligonucleotides. The PCR products were separated on 1% agarose gel electrophoresis and eluted. The 500 bp fragment obtained from the first PCR and the 350 bp fragment obtained from the second PCR were combined by a third PCR using the Near-up and the *Nhe*I-rev oligonucleotides, yielding an 800 bp NADP-MDH cDNA fragment containing the D101N mutation. The fragment was digested by *Nco*I and *Nhe*I restriction endonucleases. The whole cDNA bearing the mutation was obtained by exchanging the *Nco*I–*Nhe*I fragment of the MDH cDNA cloned in pUC9 for the fragment obtained by PCR. The *Nco*I–*Bam*HI fragment of the D101N mutated MDH cDNA was transferred to a pET vector for production of the modified protein.

The cDNA sequence was controlled at each cloning step, according to the Sanger sequencing method using the T7 kit from Amersham [14].

2.3. D101N NADP-MDH production and purification

The experimental procedures for the expression of the mutated NADP-MDH cDNA, cloned in pET vector, in BL21 *E. coli* strain and the preparation of soluble protein extracts from transformed bacteria were the same as described previously [5,8]. Purification of this mutant consisted first of an ammonium sulphate fractionation between 35 and 60% saturation in 20 mM sodium phosphate buffer, pH 7.2, 1 mM EDTA (PE buffer) and a dialysis overnight against 100 volumes of PE buffer. The fractionated extract was then loaded on a DEAE-Sephacel column (25×3 cm), washed extensively with PE buffer, and eluted with a linear gradient (2×250 ml) of 0–0.6 M NaCl in PE buffer. The active fractions were pooled and directly loaded on a Matrex Red A column (15×1.5 cm). After washing with PE buffer, the enzyme was eluted with a linear gradient (2×250 ml) 0–3 M NaCl in PE. Fractions enriched in mutant NADP-MDH were pooled, dialysed overnight against 100 volumes PE buffer and concentrated to 1–1.5 mg/ml on an Amicon ultrafiltration cell, using a YM10 membrane. Further purification was performed using an HPLC Mono Q ion exchange column. After loading the protein, elution was achieved by a 30 min linear gradient of 0–0.5 M NaCl in 30 mM Tris–HCl buffer, pH 7.9, at a flow rate of 1 ml/min. The fractions containing D101N MDH were concentrated to 2 mg/ml on an Amicon Centricon concentrator 30. Their purity was checked by vertical SDS–PAGE following the method of Schägger and Von Jagow [15]. The protein bands were visualised by Coomassie brilliant Blue staining.

Recombinant WT enzyme and a deletion mutant lacking the first 33 amino acids at the N-terminus (ΔN mutant) were produced in *E. coli* and purified as described previously [8].

2.4. Western blotting

The procedure was essentially as described previously [16]. Proteins were separated by vertical SDS–PAGE, then electrotransferred onto a nitrocellulose membrane overnight at 15 V and 4°C, in 25 mM Tris–HCl, 150 mM Glycine, 20% Ethanol. The proteins were detected using a rabbit polyclonal antibody against NADP-MDH, and visualised by peroxidase activity coupled to an anti-rabbit IgG. The D101N MDH was either analysed in dissociating reducing conditions using β-mercaptoethanol, or in dissociating non-reducing conditions, by omitting the reductant. NADP-MDH purified from sorghum leaves was used as a reference.

2.5. Molecular mass determination by size exclusion chromatography

Gel filtration experiments were performed using a TSK 3000 SW column (300×7.5 mm). After loading the protein, the elution was performed with a 50 mM phosphate buffer pH 7.2 containing 0.2 M NaCl. Proteins with known molecular masses were used as standards.

2.6. Enzyme activity assay and kinetic parameter determinations

Enzymes were activated at 25°C in 100 mM Tris–HCl, pH 7.9 buffer, with 20 μM *E. coli* thioredoxin reduced by 10 mM DTT. MDH activity was measured on activated aliquots, at 30°C, by following the decrease in absorbance at 340 nm, in a standard assay mixture (1 ml) containing 100 mM Tris–HCl, pH 7.9, 780 μM oxaloacetate and 140 μM NADPH.

The kinetic parameters were determined on pre-activated enzymes, by varying simultaneously the concentration of each substrate in the assay mixture.

3. Results

3.1. Involvement of aspartate 101 in NADP-MDH dimerisation

After a conventional MDH protein purification (anion exchange on DEAE Sephacel and affinity chromatography on Matrex Red A), the purity can be further improved by a Mono Q HPLC chromatography step. Whereas the WT protein usually yields a single peak upon elution from this chromatographic support, the D101N protein splits into two quantitatively equivalent peaks on the basis of the A_{280} absorbance (data not shown). The retention times of these two fractions were respectively 25 and 29 min. Each required a pre-activation by reduced thioredoxin to display MDH activity. In order to determine the molecular mass of the NADP-MDH contained in each fraction, they were loaded separately on a size exclusion column. The results are shown in Fig. 1. The calibration curve shows the relative retention times of molecular mass marker proteins. Sorghum NADP-MDH was used as a control: it has been previously demonstrated that this protein is a dimer [8]. The D101N MDH fraction eluting at 29 min and the dimeric sorghum NADP-MDH displayed similar apparent molecular masses, whereas the fraction eluting at 25 min corresponded to a monomeric D101N MDH form.

3.2. SDS–PAGE electrophoresis analysis of the monomeric and dimeric forms of the D101N mutant

In order to ascertain whether the monomeric and dimeric forms of NADP-MDH were present in the MDH preparation before HPLC separation and with the additional aim of unravelling the nature of the chemical interaction between the subunits of the dimeric D101N mutant, the D101N MDH partially purified by conventional chromatography techniques was analysed by Western blotting after SDS–PAGE electrophoresis either in the presence or in the absence of mercaptoethanol (Fig. 2). Under reducing conditions, a single band was observed, corresponding to a molecular mass of 40 kDa. In the absence of reductant, the blot showed two bands, one corresponding to a 40 kDa protein, the other to an 80 kDa

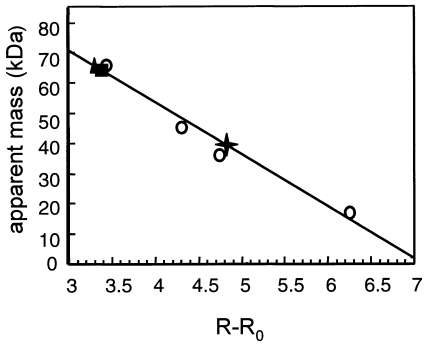

Fig. 1. Calibration curve for the apparent molecular mass determination by size exclusion chromatography. The calibration was performed using myoglobin (17 kDa), ferredoxin-NADP-reductase (36 kDa), ovalbumin (45 kDa) and serum albumin (66 kDa). Calibration proteins are represented by ○. The symbols used for the different MDH proteins are: ▲ for sorghum NADP-MDH, +: for the monomeric D101N, ■ for the dimeric D101N.

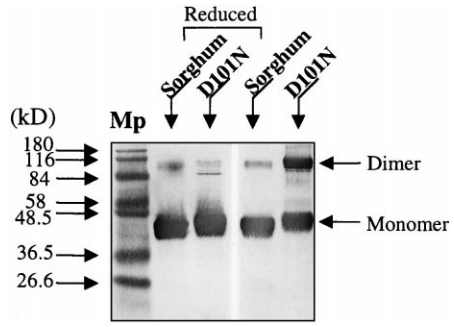

Fig. 2. Western blot of the partially purified D101N mutant and of the WT NADP-MDH. The samples loaded on SDS–PAGE either contained β-mercaptoethanol (reducing conditions) or not.

protein. Hence before the HPLC purification step, the MDH preparation is a mixture of monomeric and dimeric forms, and the two subunits of the dimer are linked by an interchain disulfide bridge.

3.3. Activation kinetics of the D101N mutant by thioredoxins

The activation kinetics of both forms of the D101N MDH were determined. The activation kinetics of the WT recombinant protein and of the ΔN mutant are given for comparison (Fig. 3). The monomeric form of the D101N mutant was activated much more rapidly than the WT protein, reaching the maximum activity within only 1 min instead of 10 min for the WT enzyme. It was found that the ΔN mutant, which was previously shown to be monomeric [8] displayed similar activation kinetics as the monomeric D101N mutant. In contrast, the dimeric D101N MDH form was activated more slowly, within ca. 10 min, much like the WT MDH (graph not shown for clarity: the activation kinetics can be roughly superimposed with those of the WT enzyme).

3.4. Kinetic parameters of the monomeric D101N mutant NADP-MDH

To obtain an active NADP-MDH, the enzyme must be pretreated with reduced thioredoxin in order to reduce regulatory disulfide bridges. This treatment converted the totality of the dimeric form of the D101N mutant into monomers (data not shown). Hence, the reduced form of the mutant is always a monomer, whereas the WT enzyme remains a dimer upon activation [11]. The kinetic parameters were determined

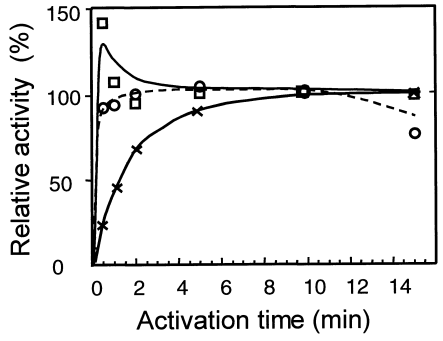

Fig. 3. Activation kinetics comparison between the D101N monomeric mutant □ and the WT MDH × or the ΔN mutant MDH ○. The activation kinetics for the dimeric D101N mutant could be roughly superimposed on the activation kinetics of the WT MDH, hence are not represented for readability.

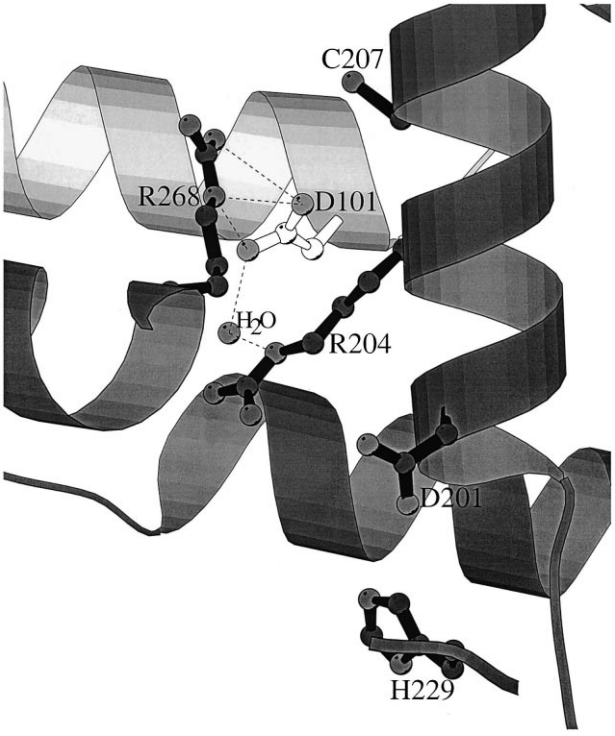

Fig. 4. Position of the mutated Asp-101 at the dimer interface in the crystal structure. Asp-101 (D101, single letter code is used) is situated in what has been shown to be a highly sensitive area, both for dimer contacts and for catalytic activity. D101 is connected to the neighbouring subunit in the dimer (here coloured white and grey, respectively) via a number of interactions. In addition to the electrostatic interaction there are three direct hydrogen bonds between D101 and R268. There is also one indirect hydrogen bond via a water molecule to R204, which is involved in coordination of the substrate in the active site. Also shown are the two catalytic residues D201 and H229. Situated in the same α-helix is also C207, the only cysteine exposed on the surface and free to make the disulfide bridge between the observed disoriented dimers. The structural data are taken from Johansson et al. [3] (PDB file name: 7 mdh).

accordingly on the fully activated monomeric D101N fraction. The results are shown in Table 1. The values for the WT recombinant NADP-MDH and the ΔN mutant are given for comparison. There is a striking loss in the overall catalytic efficiency of the D101N mutant compared to the WT NADP-MDH and ΔN mutant. This loss is due to a decrease in the affinity for each of the substrates and also to a lower catalytic turn over.

4. Discussion

4.1. Role of aspartate 101 in dimerisation

The present work demonstrates the involvement of the negative charge of Asp-101 in the stabilisation of the dimeric structure of NADP-MDH. Indeed, the HPLC and SDS–PAGE analyses reveal that the replacement of this negatively charged residue by the neutral asparagine led to a striking shift in the dimer–monomer equilibrium towards the monomeric form. The SDS–PAGE analysis of the monomeric and dimeric D101N forms in the presence or in the absence of a reductant shows that in the D101N dimeric form the two subunits are linked by a disulfide bridge. An inter-subunit disulfide bridge has never been observed in NADP-MDH,

Table 1
Kinetic parameters of the WT, ΔN and D101N NADP-MDH

	D101N mutant	WT MDH	ΔN MDH
K_m NADPH (μM)	235	50	39
K_m OAA (μM)	707	30	22
$k$cat (s^{-1})	7.5	600	671
$k$cat/(K_m OAA × K_m NADP) (M^{-2} s^{-1})	4.5×10^7	$40\,000\times10^7$	$78\,243\times10^7$

neither in the enzyme purified from sorghum leaves, nor in the recombinant WT protein [11]. Thus it can be considered as an artefact, probably due to the abundance of free monomers which undergo a partial oxidation during the protein purification and/or storage. The fact that it does not form in the WT enzyme suggests that in the mutated enzyme cysteines which are distant in the WT protein come in close contact to each other. Cysteines 24 and 29 of the N-terminal extension are known to be linked in a disulfide bridge in the oxidised WT protein, thus it is highly unlikely that they would be involved in an inter-subunit disulfide. The same consideration applies to the C-terminal Cys-365 and 377 which are linked in the C-terminal disulfide. Among the four internal cysteines, three are known to be buried in the oxidised WT enzyme [3]. The fourth one, Cys-207, has been shown to form mixed disulfides with thioredoxin and disulfide-cross-linked dimers with the monomeric N-terminal deletion mutant [17]. Hence this cysteine is a good candidate for the observed disulfide cross-linking of the D101N mutant. The monomeric state of this mutant would make it susceptible to such a cross-linking, as seen with the N-terminal deletion mutant. One can imagine that the Cys-207 of each subunit, situated in diametrically opposite directions in the WT dimer, can enter in contact in the freely moving monomers, forming disoriented disulfide-cross-linked dimers. The basic state of the D101N mutant can thus be considered to be monomeric. The negative charge of Asp-101 seems to play a prominent role in the stability of the dimer and more specifically in the orientation of one monomer with respect to the other one, maintaining the Cys-207 of the two subunits too far apart to form a disulfide in the WT protein. Moreover, the Asp-101 residue in NADP-MDH seems to play a similar role as the His-56 residue of mitochondrial NAD-MDH. Indeed, these residues are found at the same position in the primary structures of the proteins [3], as well as in their 3D structures [3,18]: they are both located at the C-terminal end of the αC helix and oriented towards the outside of each subunit, a situation rendering them prone to make inter-subunit interactions. However, the nature of the interactions is obviously different. From the crystal structure of NADP-MDH [3] one can determine that the negatively charged Asp-101 certainly makes several interactions with positively charged residues of the neighbouring subunit. Asp-101 is indeed involved in a salt bridge with Arg-268 (Fig. 4). There are three direct hydrogen bonds to Arg-268 and also one indirect bond via a water molecule to Arg-204, a residue known to be involved in the coordination of the substrate in the active site. In the mitochondrial NAD-MDH [18], His-46 is directly hydrogen-bonded to Arg-152 [12], the residue corresponding to Arg-204 in NADP-MDH.

The mutation of Asp-101 to Asn results in a loss of the negative charge and thus a loss of the salt bridge to Arg-268. In addition, it disturbs the hydrogen-bonding pattern in the interactions between subunits because of the loss of one single hydrogen bond. It is surprising that such a small change in bonding potential causes such a dramatic change that leads to a total loss of dimeric interactions. In comparison, the H46L mutation of the mitochondrial enzyme results in a strengthening of the dimeric interactions, probably due to the possibility for the Leu to make hydrophobic interactions instead of the pH-dependent hydrogen bond to Arg-152.

4.2. Aspartate 101 and activation kinetics

The D101N monomeric mutant has the same accelerated activation time-course as the previously described ΔN monomeric mutant [8]. This result confirms that the slow activation kinetics described for WT NADP-MDH is linked to its dimeric state, as previously proposed [11]. The disulfide cross-linked dimer of the mutated protein displayed activation kinetics similar to those of the WT enzyme (data not shown). This suggests that the slow activation kinetics are related to the dimeric form of the enzyme. However, the activation mechanism should be somewhat different in the WT enzyme and in the D101N mutant: in the latter case, an intersubunit disulfide needs to be reduced during the course of activation, with a gradual change in the aggregation state of the enzyme towards the formation of monomers.

4.3. Role of aspartate 101 in catalysis

The comparison of the kinetic parameters of the activated D101N mutant with those of the activated ΔN mutant indicates that the Asp-101 residue is also important for the catalytic activity of the enzyme. The affinities of the D101N mutant for NADPH and oxaloacetate are markedly lower than those of the WT and ΔN proteins (K_m increased five-fold and 20-fold respectively) and the catalytic constant is decreased 100-fold. These results are very similar to those obtained in yeast mitochondrial MDH with the H46L mutant [12]. In our previous activation model [5], we proposed that the acquisition of the catalytic competence of the active site during activation would involve a modification of the interaction between subunits based on the electrostatic interaction between Asp-101 of one subunit with the Arg-204 of the other one which would change the distance between the two arginines known to bind oxaloacetate in NAD-dependent MDH, namely Arg-204 and Arg-134. Now we know from the 3D-structure that Asp-101 of the oxidised NADP-MDH interacts more directly with Arg-268 which does not belong to the active site. However, there is also an indirect hydrogen bond via a water molecule to Arg-204. This indirect effect could help to position and stabilise Arg-204 with respect to the substrate, thus yielding a higher binding constant and catalytic efficiency. Alternatively, it cannot be excluded that upon reduction of the enzyme the active site undergoes a conformational change allowing Asp-101 to interact more directly with Arg-204. However, the loss of such an interaction cannot account for the unfavourable effect of the mutation on catal-

ysis: indeed the monomeric ΔN mutant has a better catalytic efficiency than the WT enzyme. Thus it must be concluded that Asp-101, in addition to being essential for the dimerisation, is also an intrinsically important residue for the catalytic activity of the enzyme. This is a very striking finding, considering that the distances between Asp-101 to both the NADP(H) (20 Å) and oxaloacetate (30 Å) within its own monomer are very large. Such distances naturally exclude any kind of direct interactions and the function of Asp-101 in catalysis must be considered as a problem yet to be solved.

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