One Site Mutation Disrupts Dimer Formation in Human DPP-IV Proteins*

DPP-IV is a prolyl dipeptidase, cleaving the peptide bond after the penultimate proline residue. It is an important drug target for the treatment of type II diabetes. DPP-IV is active as a dimer, and monomeric DPP-IV has been speculated to be inactive. In this study, we have identified the C-terminal loop of DPP-IV, highly conserved among prolyl dipeptidases, as essential for dimer formation and optimal catalysis. The conserved residue His750 on the loop contributes significantly for dimer stability. We have determined the quaternary structures of the wild type, H750A, and H750E mutant enzymes by several independent methods including chemical cross-linking, gel electrophoresis, size exclusion chromatography, and analytical ultracentrifugation. Wild-type DPP-IV exists as dimers both in the intact cell and in vitro after purification from human semen or insect cells. The H750A mutation results in a mixture of DPP-IV dimer and monomer. H750A dimer has the same kinetic constants as those of the wild type, whereas the H750A monomer has a 60-fold decrease in $k_{cat}$. Replacement of His750 with a negatively charged Glu (H750E) results in nearly exclusive monomers with a 300-fold decrease in catalytic activity. Interestingly, there is no dynamic equilibrium between the dimer and the monomer for all forms of DPP-IVs studied here. This is the first study of the function of the C-terminal loop as well as monomeric mutant DPP-IVs with respect to their enzymatic activities. The study has important implications for the discovery of drugs targeted to the dimer interface.

Dipeptidyl peptidase IV (DPP-IV,1 also known as CD26) (EC 3.4.14.5) is a well documented drug target for the treatment of type II diabetes (1). It is a serine protease involved in the \textit{in vivo} degradation of two insulin-sensing hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (2, 3). Either inhibiting the enzymatic activity of DPP-IV in various animal models or knocking out DPP-IV in mice and rats prolongs the half-lives of these two insulin-sensing hormones, increases insulin secretion and improves glucose tolerance (4–11). Hence inhibition of DPP-IV may be effective in the treatment of type II diabetes. Understanding the catalytic mechanism of DPP-IV is thus essential to discovering inhibitors for the treatment of the disease.

DPP-IV belongs to the prolyl oligopeptidase (POP) family, a subfamily of serine proteases (12, 13). This class of prolyl peptidases includes DPP-IV, prolyl oligopeptidase (POP), DPP-II, DPP8, DPP9, and fibroblast activation protein (FAP) (12, 13). Unlike classic serine proteases, the POP family of enzymes is highly selective toward peptides that have a proline residue at the penultimate position (18). The x-ray structures of DPP-IV and POP have shed light on the catalytic mechanisms, which differ significantly from those of the classic serine proteases, such as trypsin and subtilisin (12, 14–18). DPP-IV consists of two domains, the \(\alpha/\beta\) hydrolase domain and the \(\beta\)-propeller domain, with the active site inbetween (14–17). The substrate specificity of DPP-IV is dictated by a proline-binding pocket and a Glu\(^{285}\)Glu\(^{286}\) motif at the active site (14–17). Only small size peptides are hydrolyzed by this class of enzymes because of the unique propeller structure and/or side opening substrates used to access the active site (14–17). Among the shared properties, the most obvious difference between POP and DPP-IV is the relationship of catalytic activity with respect to its quaternary structure. POP exists in solution as a monomer and is active in such a form (15). In contrast, DPP-IV is active only as a dimer or oligomer, and monomeric DPP-IV is speculated to be inactive, even though DPP-IV monomer has never been isolated and demonstrated to be inactive (14, 19).

Based on the crystal structures of DPP-IV, two loops are located in the dimer interface and were proposed to be involved in dimer interaction, the C-terminal loop at the \(\alpha/\beta\) hydrolase domain and the propeller loop extended from strand 2 of the fourth blade in the \(\beta\)-propeller domain (14, 16, 17) (Fig. 1A). The C-terminal loop of DPP-IV consists of the last 50 amino acid residues with two \(\alpha\)-helices (amino acids 713–725 and 745–763) and one \(\beta\)-sheet (amino acids 726–744) interacting with the same region from the other monomer across a 2-fold axis (Fig. 1B). Both hydrophobic and hydrophilic interactions have been proposed to be responsible for dimer formation (12–15). This loop is highly conserved among the family of DPP-IV-containing prolyl dipeptidases (Fig. 2), though the functional
In this article, the quaternary structures and catalytic activities were studied and compared among endogenous DPP-IV from human semen, recombinant wild-type and mutant DPP-IVs expressed in baculovirus-infected insect cells. The role of the highly conserved C-terminal loop for dimerization was investigated. For the first time, we have isolated and characterized the biochemical properties of monomeric mutant DPP-IV proteins altered at residue His750 of the loop, which is highly conserved among DPP-IV-containing prolyl dipeptidases (Fig. 2).

**EXPERIMENTAL PROCEDURES**

**Materials**—The enzyme substrate H-Gly-Pro-pNA and dipeptidase Gly-Pro were purchased from Bachem. Fetal bovine serum was from Hyclone. Lipofectin and the insect culture media, Grace and Express Five media, were from Invitrogen. Human liver cDNA library and linear viral vector were from Clontech. The ECL Western detection kit was from Hyclone. Lipofectin and the insect culture media, Grace and Express XL-A analytical ultracentrifuge with an An60Ti rotor as described (25). Sednterp version 1.07 program is used to obtain solvent density, viscosity, Stokes’ radius (Rs) and anhydrous frictional ratio (f/f0).

**Analytical Ultracentrifugation**—DPP-IV proteins at concentrations of around 0.1 to 0.2 mg/ml (1.2–2.3 μM) were used for AUC analysis with either PBS, high salt (100 mM Tris-HCl, 50 mM NaCl, 0.5 mM Na2SO4, pH 7.5) or low salt (100 mM Tris-HCl, 50 mM NaCl, pH 7.5) buffers as indicated. Buffers were exchanged using an Amicon device and DPP-IV proteins were allowed to equilibrate for at least 4 h or longer as indicated in the text at 25 °C after buffer changes. The sedimentation coefficients (S) of the enzyme were estimated by a Beckman-Coulter XL-A analytical ultracentrifuge with an An60Ti rotor as described (25). Sedimentation velocity analysis was performed at 40,000 rpm at 25 °C with standard double sector aluminum centerpieces. The UV absorption of the cells was scanned every 5 min for 4 h. Sedimentation equilibrium was performed at 20 °C with six-channel open centerpieces and then centrifuged at 12,000 rpm for 12 h. The data from both sedimentation velocity and sedimentation experiments were analyzed with the SedFit version 8.7 program to obtain molecular weights and sedimentation coefficients (25). Sedneterp version 1.07 program is used to obtain solvent density, viscosity, Stokes’ radius (Rs) and anhydrous frictional ratio (f/f0).

**Dilution Experiment**—Enzyme concentrations ranging from 200 to 1 pM were used in the dilution experiments. The experiments were carried out with consecutive 2-fold dilutions in PBS containing 0.1% bovine serum albumin and 1 mM EDTA. The solution after dilution was incubated at 25 °C for 16 h to ensure attainment of dimer-monomer equilibrium. The reaction was initiated by adding the substrate H-Gly-Pro-pNA at a final concentration of 10 μM for both wild-type DPP-IV and H750A proteins. The initial rate of the reaction was recorded and converted to specific activity.
RESULTS

Human DPP-IV Protein Is a Dimer in Intact Cells and in Vitro—From the crystal structures, the human recombinant DPP-IV was shown to be a homodimer whereas DPP-IV purified from porcine kidney is a homotetramer (14, 16, 17, 26). In addition, previous studies showed that purified DPP-IV proteins from various sources migrated at sizes corresponding to either dimer or tetramer/oligomer according to gel filtration experiments (19, 27–30). To determine the physiologically relevant oligomerization state of DPP-IV, we performed chemical cross-linking in the DPP-IV-containing Caco-2 cells. The chemical cross-linker used was DTSP, a primary amine-specific cross-linker with moderate chain length. As shown in Fig. 3A, DPP-IV could form a dimer (240 kDa) in intact cells, twice the size of the monomer (110 kDa) (Fig. 3A, lane 2). The cross-linker DTSP is specific, because the addition of the DTT abolishes dimer formation (Fig. 3A, lane 1). The formation of dimer is DTSP-dependent since in the absence of DTSP, no dimer formation was observed (Fig. 3A, lanes 3 and 4).

Next we determined whether endogenous DPP-IV purified from human semen (sDPP-IV) forms dimers in vitro. The purified protein was quite pure as demonstrated by SDS-PAGE (Fig. 3B, lane 1). By measuring its kinetic constants ($k_{cat}$ and $K_m$ values), we confirmed that purified sDPP-IV was as active as reported previously (Table I) (31). On a native gel, sDPP-IV runs predominantly as a dimer of about 200 kDa with the presence of minor but higher molecular mass species (Fig. 3C, lane 1). It elutes at a position corresponding to a 400 kDa protein with a Stokes' radius of 5.9 nm, determined by gel filtration chromatography (Fig. 4A and Table II). Cross-linking of the purified protein in vitro showed that the protein is dimeric with a mass of 250 kDa (data not shown). We then used AUC to determine the hydrodynamic properties of sDPP-IV. As shown in Fig. 5A, sDPP-IV is undoubtedly homodimeric with a sedimentation coefficient of 9.1 S (Table II) and a molecular mass of 225 kDa. Notably, there is only a single peak corresponding to the dimer in the AUC experiment, suggesting that the dimer is the predominant form under the conditions tested. For sDPP-IV, the value of the anhydrous frictional ratio $f/f_0$ is 1.4, indicating that the protein is non-spherical. Therefore, gel filtration does not provide an accurate measurement of sDPP-IV's quaternary structure and molecular weight, because of the protein's non-globular shape. The aberrant mobility in gel filtration was also observed in previous studies with DPP-IV proteins purified from either human fibroblast cells or urine (29, 32).

Properties of the Baculoviral-expressed DPP-IV Proteins—Determination of the residues important for dimer formation necessitated the generation of mutant DPP-IVs. We chose baculoviral-infected insect cells to express both wild-type and mutant DPP-IV proteins for the in vitro biochemical studies. Whether the recombinant DPP-IV (rDPP-IV) is also dimeric in solution and has comparable biochemical properties, despite the difference in glycosylation between the endogenous sDPP-IV and rDPP-IV, were investigated.

rDPP-IV from baculoviral-infected insect cells was purified and found to be as active as endogenous sDPP-IV, based on
The experiments were repeated at least three times with similar results obtained using different batches of purified proteins. What is shown here is one representative set of the data. Substrate used for kinetic constant measurement is H-Gly-Pro-pNA. The experiments were carried out as described under “Experimental Procedures.”

| Kinetic constants of wild type and mutant DPP-IVs |
|-----------------------------------------------|
| PBS buffer | | High salt buffer |
| kcat (s⁻¹) | Km (μM) | kcat/Km | kcat (s⁻¹) | Km (μM) | kcat/Km |
| sDPP-IV | 73 | 96 | 0.76 |
| rDPP-IV | 87 | 90 | 0.97 |
| H750A monomer | 31 | 77 | 0.40 |
| H750A dimer | 1.4 | 64 | 0.02 |
| H750E | 2.6 | 956 | 0.003 |
| H750E | ND | ND | ND |
| H750 E | 3.7 | 181 | 0.31 |
| ND, not determined. |

**FIG. 4. Gel filtration profiles of DPP-IV proteins.** A, sDPP-IV; B, rDPP-IV; C, H750A; D, H750E. Inset, calibration of the gel filtration column with the Stokes radii of the protein markers was described under “Experimental Procedures.”

**H750 Is Important for Dimer Formation and Stability**—One important interaction between two monomers of DPP-IV is provided by the C-terminal loop located at the α/β hydrolase domain (14, 16, 17) (Fig. 1, A and B). Based on the sequence alignment of the prolyl dipeptidases presented in Fig. 2, the highly conserved C-terminal loop might play an important role in the dimerization of DPP-IV and other prolyl dipeptidases.

**TABLE I**

The size difference between sDPP-IV and rDPP-IV in SDS-PAGE, native gel, gel filtration and sedimentation experiments, reflects the difference in the extent and nature of the glycosylation and the non-spherical nature of the dimeric proteins. This is also consistent with the difference observed in Stokes’ radii between these two wild-type proteins in AUC (Table II).
filtration experiment to separate these two forms of H750A before subjecting them separately to sedimentation equilibrium analysis and the measurement of the enzymatic activities. As shown in Fig. 6, interestingly, both dimer and monomer maintained their subunit compositions without converting into monomer or dimer, respectively, after incubation at room temperature for up to 48 h. This indicates that a dynamic equilibrium between dimer and monomer of H750A is extremely slow or non-existent.

The kinetic constants were measured for the monomer and the dimer of H750A after separation by gel filtration. As shown in Table I, dimeric H750A has activity similar to that of the wild-type rDPP-IV indicating that, in the absence of change in quaternary structure, the mutation did not perturb the enzymatic activity. However, monomeric H750A has a 60-fold decrease in the $k_{cat}$ but a similar $K_m$ value. Therefore, the quaternary structure of enzymes correlates with the enzymatic activities since both monomeric H750A and H750E have much lower catalytic activities compared with those of the dimeric rDPP-IV or H750A.

Since the sedimentation velocity depends on both size and shape of the protein, we carried out a sedimentation equilibrium analysis and the measurement of the enzymatic activities. As shown in Table II, the experiments were repeated at least twice with similar results obtained using different batches of purified proteins. What is shown here is one representative set of the data. The predicted monomeric $M_r$ of sDPP-IV and rDPP-IV without glycosylation is 85,246 and 84,371, respectively.

**Table II: Hydrodynamic properties of wild type and mutant DPP-IVs**

| Protein          | PBS buffer | High salt buffer |
|------------------|------------|-----------------|
|                  | sDPP-IV    | rDPP-IV         |
| Stokes' radius ($R_s$) (nm) | 5.9$^a$ | 5.6$^c$ | 5.7$^c$ | 4.6$^c$ | 4.6$^c$ | 4.9$^b$ | 4.9$^b$ | 3.7$^b$ | 3.7$^b$ |
| Sedimentation coefficient ($s_{20,w}$) (S) | 9.1$^a$ | 5.9$^b$ | 5.1$^b$ | 4.1$^b$ | 4.0$^b$ | 4.9 | 5.0 | 3.3 | 3.3 |
| Molecular weight ($M_r$) | 225k$^c$ | 187k$^c$ | 186k$^c$ | 98k$^c$ | 98k$^c$ | 154k | 158k | 78k | 79k |
| Anhydrous frictional ratio ($f/f_o$) | 1.4 | 1.4 | 1.4 | 1.3 | 1.3 | ND | ND | ND | ND |

$^a$ The values of the Stokes' radii were obtained from gel filtration experiments.

$^b$ The values of the Stokes' radii were obtained from sedimentation velocity experiments.

$^c$ The values determined were from sedimentation equilibrium experiments.

$^d$ ND, not determined.

**Fig. 5. Sedimentation velocity analysis of DPP-IV proteins.** A, sDPP-IV; B, rDPP-IV; C, H750A; D, H750E. The three panels in each experiment represent the trace of absorbance at 280 nm during the sedimentation, the residues of the model fitting, and the sedimentation coefficient distribution of all species.
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Fig. 6. AUC analysis of separated H750A proteins. A, dimeric H750A after gel filtration; B, monomeric H750A after gel filtration.

rium analysis to determine unambiguously the molecular masses for all forms of DPP-IVs studied here. Summarized in Table II, for sDPP-IV, rDPP-IV, H750A dimer, H750A monomer and H750E, the molecular masses determined are 225, 187, 186, 98, and 98 kDa, respectively. The values obtained are comparable with those from sedimentation velocity experiments, consistent with no dynamic equilibration. All the mutant proteins analyzed have the $f_{20w}$ values of 1.3–1.4, indicating that they are all non-spherical in shape.

Dilution Experiment—Because the monomer and dimer of H750A did not equilibrate under the conditions tested, we wanted to know whether dilution of the enzyme to very low concentrations would facilitate the dissociation of the dimer into monomer. The dilution method has been used to study the dimer-monomer equilibrium of several herpes viral proteases with $K_d$ values in the nanomolar range (33–35). If the monomeric DPP-IV has a very low activity as observed for monomeric H750A and H750E, dilution of the protease to a concentration near or below the $K_d$ value might result in the formation of low activity monomeric DPP-IV. As a result, the specific activity measured will be decreased (33–35). We did not observe decreased specific activity for either sDPP-IV or rDPP-IV, even at a concentration as low as 1.6 nM (Fig. 7, A and B). Similarly, the specific activity of H750A protein was also constant over the range of 200–1.6 nM (Fig. 7C). These results along with sedimentation equilibrium experiments (Fig. 6) are consistent with the lack of a dynamic equilibrium between monomeric and dimeric H750A and the small $K_d$ of wild-type DPP-IV dimer.

Effects of Salts, Active Site Inhibitor, and Dipeptide Product on DPP-IV Structure—A high concentration of anti-chaotropic salts, such as sulfate, phosphate, and citrate, enhances the stability of dimer for several herpes virus proteases (34, 36–38). High salt buffer (0.5 M sodium sulfate) has been used to probe the dimer-monomer equilibrium for dimeric HCMV protease (36). We determined whether the same high salt (0.5 M sodium sulfate) had any effect on the quaternary structure of DPP-IVs studied in this article. As shown in Fig. 8 and Table II, high salt did not change the composition of the subunits for any DPP-IV, since the molecular masses and Stokes’ radii measured by sedimentation velocity still correspond to dimer, mixture of dimer-monomer and monomer for rDPP-IV, H750A and H750E, respectively, similar to results obtained in PBS buffer. However, they all show significant global conformational changes as indicated by dramatic shifts in sedimentation coefficients (Fig. 8 and Table II). The $S$ values for the dimeric forms of rDPP-IV and H750A change from 8.4 to 5.0 S, and the monomeric H750A and H750E from 5.5 to 3.3 S (Fig. 8 and Table II). Interestingly, the kinetic constants of these DPP-IVs in high salt are comparable to the corresponding ones in PBS buffer with a slight increment in the $K_m$ for rDPP-IV and H750A (Table I). This result suggests that the interaction between the monomers of DPP-IV is quite different from that in HCMV protease. The subunit composition and activity of DPP-IVs were also studied in low salt buffer. We did not find any differences in either AUC analysis or catalytic activities for rDPP-IV, H750A, or H750E protein, as compared with those in PBS buffer (data not shown).

To determine whether substrate could induce dimerization of DPP-IV, we performed AUC for H750A in the presence of the proline-mimetic inhibitor and Gly-Pro dipeptide product, because the substrate Gly-Pro-pNA is cleaved by the enzyme. The proline-mimetic inhibitor, 1-(2-amino-2-cyclohexyl-acyl)-2-cyano-(S)-pyrrolidine, targets to the active site and has an $IC_{50}$ value of less than 50 nM (6). The monomeric forms of H750A and H750E do not shift to dimer in the presence of either dipeptide product or the inhibitor in either PBS or high salt buffer (data not shown).

DISCUSSION

Dimerization is an important way to regulate the activities of many proteins, such as herpes viral and retroviral proteases, SARS 3C protease, caspase 9, and STATs (39–43). In this article, we have studied the catalytic activity of DPP-IV with respect to its quaternary structure. Despite a much lower extent of glycosylation, human DPP-IV expressed in insect cells has similar biochemical properties, catalytic activities, and dimer structure, compared with those of the endogenous human semen DPP-IV. Using cross-linking and analytical ultracentrifugation (AUC), we showed that DPP-IV is dimeric both in vivo and in vitro.

We showed that the C-terminal loop of DPP-IV is essential for dimer formation and optimal catalytic efficiency. As the dimer interface formed by the C-terminal loop is 2-fold symmetric (Fig. 1, A and B), a single mutation is therefore functionally equivalent to double mutations in this dimeric enzyme. This is the first study where monomeric DPP-IVs, H750A, and H750E, were generated, purified to homogeneity, and studied. Detailed kinetic analysis showed that monomeric H750A has a 60-fold drop of the $k_{cat}$ with no change in the $K_m$, whereas both $k_{cat}$ and $K_m$ of H750E are remarkably changed, with a more severe effect on $k_{cat}$ (30-fold reduction) than $K_m$ value (10-fold increment). The result is particularly interesting since it reveals that the monomers of DPP-IV are not void of activity as previously speculated. Instead, much lower activities compared with the dimeric DPP-IV are associated with the monomeric DPP-IVs. The difference in the $K_m$ between these two monomeric DPP-IV mutant proteins, H750A and H750E, might be caused by a charge effect, affecting the conformation of the active site and/or the binding of the substrate. The data also suggest that the structure of DPP-IV is sensitive to packing interactions around His$^{750}$. His$^{750}$ is located in the vicinity of several bulky hydrophobic residues, such as Val$^{726}$, Val$^{728}$, and Phe$^{730}$, with the exception of the charged residue Asp$^{729}$. The carboxyl of Val$^{728}$ is within hydrogen bonding distance of the imidazole ring of His$^{750}$ as marked on Fig. 1C. The drastic effect of H750E on disrupting the dimeric DPP-IV to monomer might be caused by charge repulsion generated between Glu$^{750}$ (H750E) of one monomer and Asp$^{729}$ of the other (Fig. 1C). On the other hand, generation of the monomeric H750A suggests that the interaction mediated by the imidazole ring with the neighboring residues are crucial for dimer stability, further stressing the critical role of this residue for the C-terminal loop.

The interaction between the C-terminal loops of DPP-IV is most likely to hold the catalytic triad and the active site in an optimal position for catalysis. The formation of the monomer...
upon losing the dimer interaction might result in the disorientation of the loop. Since two of the three triad residues (Asp\(^{708}\) and His\(^{740}\)) are located on the C-terminal loop and close to the actual dimerization interface (17), the optimal alignment of the triad needed for catalysis, the conformation of the substrate binding pocket or/and the position of an oxyanion hole might be affected upon monomer formation. The studies on dimeric HCMV and HIV proteases have revealed that upon the introduction of the mutation on the C-terminal loop emphasizes the importance of the C-terminal loop in dimer formation and maintenance. In the DPP-IV-containing prolyl dipeptidase family (Fig. 2), it is not clear whether other members adopt similar quaternary structures similar to that of DPP-IV. Because the C-terminal loop of DPP-IV is very highly conserved (Fig. 2), it is likely that this loop is a general dimerization motif used by other members of the prolyl dipeptidases.

We have identified the His\(^{750}\) residue, completely conserved as well among different prolyl dipeptidases, as essential for dimer formation. In addition, we found that high salt induces significant global conformational changes without affecting the subunit composition and the catalytic activities of the DPP-IVs. Therefore, salt has much less effect and is not capable of disrupting the dimer of DPP-IV to monomer or promoting dimer stability. This is contrary to HCMV protease, whose dimer is stabilized by high salt with a concomitant increase in catalytic activities (34, 36, 39, 45). Based on our data (Figs. 5–8), the interaction between the monomers in DPP-IV is much stronger than that of the HCMV protease, supported by the lack of salt-induced effect for DPP-IV.

One of the most surprising findings of this study is that there is no dynamic equilibration between the dimer and monomer of either wild-type or mutant DPP-IVs in vitro (Figs. 6 and 7). The formation of dimeric H750A by the insect cells may be assisted and promoted in vitro by chaperone proteins in the endoplasmic reticulum or by a local high concentration of the proteins during synthesis. Once dimeric H750A is formed in vivo, it does not dissociate into monomer again in vitro (Figs. 6 and 7). This indicates that there are additional interactions present in the dimer interface to compensate for the loss of the interaction by the imidazole ring of H750. The dilution experiments are consistent with the AUC experiments, indicating that there is no change of the dimer-monomer composition. This might explain the fact that up to the present time, there is no report of the introduction of the mutation on the C-terminal loop.
isolation of the monomeric form of wild-type DPP-IV. In addition, the presence of the dipeptide product or the inhibitor failed to promote the dimerization, demonstrated in this study. These data suggested that there is not sufficient activation energy to shift either monomer to dimer or dimer to the monomer form.

To address the drug resistance commonly observed with current active site inhibitors, there is an alternative approach directed toward finding novel drugs targeting protein interface inhibitors. The current active site inhibitors, there is an alternative approach directed toward finding novel drugs targeting protein interface. In this dimerization context, it is possible that we might identify a possible “binding site” at the dimer interface resulting in disrupting the active dimer to inhibit DPP-IV activity. The study presented here elucidates the reaction mechanism of DPP-IV and may facilitate the anti-diabetic drug discovery.

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