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Transient Formation of a Neutral Ubisemiquinone Radical and Subsequent Intramolecular Electron Transfer to Pyrroloquinoline Quinone in the *Escherichia coli* Membrane-Integrated Glucose Dehydrogenase†

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ABSTRACT: The membrane-bound quinoprotein glucose dehydrogenase (mGDH) in *Escherichia coli* contains pyrroloquinoline quinone (PQQ) and participates in the direct oxidation of D-glucose to D-gluconate by transferring electrons to ubiquinone (UQ). To elucidate the mechanism of ubiquinone reduction by mGDH, we applied a pulse radiolysis technique to mGDH with or without bound UQ_8. With the UQ_8-bound enzyme, a hydrated electron reacted with mGDH to form a transient species with an absorption maximum at 420 nm, characteristic of formation of a neutral ubisemiquinone radical. Subsequently, the decay of the absorbance at 420 nm was accompanied by an increase in the absorbance at 370 nm. Experiments with the PQQ-free apoenzyme showed no such subsequent absorption changes, although ubisemiquinone was formed. These results indicate that a pathway for an intramolecular electron transfer from ubisemiquinone radical at the UQ_8 binding site to PQQ exists in mGDH. The first-order rate constant of this process was calculated to be equal to 1.2×10^3 s^{-1}. These findings are consistent with our proposal that during the catalytic cycle of mGDH the bound UQ_8 mediates electron transfer from the reduced PQQ to UQ_8 pools.

Ubiquinone (UQ)[1] molecules are involved in many of the oxidation−reduction processes of respiration and photosynthesis in living cells. They are able to freely diffuse in membranes (e.g., UQ pools) or able to bind to membrane proteins in the binding pockets where they act as one- or two-electron gates (*1*). It has been shown that structurally identical UQ can have very different chemical properties when bound in different sites within a protein (*2*). For example, in the photosynthetic reaction center, Q_A acts as a tightly bound one-electron carrier between pheophytin and Q_B, whereas Q_B is a two-electron and two-proton redox component and in dynamic equilibrium with the UQ pool in the membrane (*3*). For the cytochrome bc_1 complex (complex III), two UQ/UQH_2 redox sites, Q_o and Q_i, are both in dynamic equilibrium with the UQ pool, and the coordinated redox reactions are tightly coupled to vectorial proton translocation via the proton-motive Q cycle (*4*). In *Escherichia coli* cytochrome *bo*, bound UQ_8 at the high-affinity UQ binding site (Q_H) serves as an electron gate and mediates the sequential one-electron transfer to hemes *b* and *o* from the low-affinity UQ binding site (Q_L) (*5*), where the two-electron oxidation of UQH_2 takes place. In these processes, the ubisemiquinone radical is formed as an intermediate, and appears to function as a gate for electron flow through the sequential one-electron transfer (*6*−*10*).

Membrane-bound glucose dehydrogenase (mGDH) of *E. coli*, a member of the PQQ-containing quinoprotein family (*11*, *12*), catalyzes the direct oxidation of D-glucose to D-gluconate, and concomitantly feeds electrons to ubiquinol oxidase through UQ in the respiratory chain (*13*−*15*). The mGDH in *E. coli* exists as an apoenzyme because of the absence of genes responsible for PQQ synthesis (*16*, *17*), and the exogenous addition of PQQ with a divalent cation leads to formation of the active enzyme (*18*). The mGDH is an 88 kDa monomeric protein with two distinct domains: an N-terminal hydrophobic domain and a large C-terminal periplasmic domain (*15*). The latter domain has a β-sheet propeller fold superbarrel structure that is a catalytic domain, including PQQ (*11*, *19*) and Ca^{2+} or Mg^{2+} binding sites (*16*, *17*). The UQ reduction site for the bulk UQ pool in mGDH has been shown to be located near the membrane surface (*21*), an idea which was strengthened by the findings that its C-terminal periplasmic domain, interacting peripherally with the membrane, possesses the UQ reduction site (*21*). On the basis of EPR spectra and the effects of inhibitors on UQ_2 reductase activity, we recently postulated that mGDH possesses two UQ-binding sites, Q_I for bound UQ_8 and Q_{II} for the bulk UQ pool (*22*), and it was found that a PQQ semiquinone radical was formed by the addition of D-glucose to mGDH (*22*). This suggests that bound UQ_8 accepts one electron from $PQQH_2$ to form the PQQ semiquinone radical,

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[1] Abbreviations: mGDH, membrane-bound glucose dehydrogenase; PQQ, pyrroloquinoline quinone; UQ, ubiquinone; DM, *N*-dodecyl-β-D-maltoside; e_{aq}^-, hydrated electron.

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though EPR signals of UQ_8 semiquinone were not observed. However, the mechanisms for the intramolecular electron transfer between PQQ and UQ_8 in mGDH are not well characterized.

A powerful approach to investigating electron transfer within proteins is that of pulse radiolysis through which an electron can be introduced rapidly into one redox center of an enzyme (*23*−*29*). Our previous study on cytochrome *bo* revealed that the *N*-methylnicotinamide radical mediator produced by pulse radiolysis caused rapid reduction of a bound Q_8 at the Q_H site, followed by intramolecular electron transfer to hemes *b* and *o* in the *E. coli* cytochrome *bo* (*28*). To dissect the intramolecular electron transfer process in PQQ-containing dehydrogenase, we applied pulse radiolysis to *E. coli* mGDH. Differences in the reactions after pulse radiolysis occurring in the UQ-bound apoenzyme (lacking PQQ), the UQ-bound holoenzyme, the UQ-free apoenzyme, and the UQ-free holoenzyme are reported. Here, we present the formation of a ubisemiquinone neutral radical in mGDH and the subsequent intramolecular electron transfer to PQQ.

EXPERIMENTAL PROCEDURES

Bacterial Strains. The *E. coli* K-12 strains used in this study were W3110 [IN (*rrnD-rrnE*) *rph-1*] (*30*) and YU654 (W3110 *ubiA*::*cml*) (*22*). YU654 cannot produce UQ because of a deletion in the *ubiA* gene encoding 4-hydroxybenzoate octaprenyltransferase. As described previously, these were used as host strains for purification of wild-type and UQ_8-free mGDH, respectively (*22*).

Purification of the Wild-Type mGDH and UQ_8-Free mGDH. W3110 cells harboring pUCGCD1 (*30*) and YU654 (*ubiA::cml*) cells harboring pUCGCD1 were grown in LB medium (1% bactotryptone, 0.5% yeast extract, and 0.5% NaCl) containing ampicillin (50 μg/mL) for 12 h at 30 °C with aeration (200 rpm). Cells were collected by centrifugation and washed once with saline solution and twice with potassium phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$. Washed cells were disrupted by being passed twice through a French pressure cell press at 16 000 psi. The membrane fraction was recovered by ultracentrifugation (86000*g* for 90 min). The mGDH was purified from the membrane fractions at 4 °C by column chromatography on DEAE-Toyopearl (Toso) and ceramic hydroxyapatite (Bio-Rad) using a modification of the previously reported procedure (*16*). Membrane fractions (approximately 10 mg/mL protein) were treated with 10 mM potassium phosphate buffer (pH 7.0) containing 0.04% *N*-dodecyl-β-D-maltoside (DM), and the resulting membrane fractions were subjected to solubilization for 60 min in the presence of 10 mM potassium phosphate buffer (pH 7.0) containing 0.2% DM and 100 mM KCl. The suspension was centrifuged at 86000*g* for 90 min, and the resulting supernatant was dialyzed against the same buffer without DM. The dialysate was applied to a DEAE-Toyopearl column (1 mL bed volume; approximately 10 mg of protein), followed by a ceramic hydroxyapatite column (5 mL bed volume; approximately 5 mg of protein) as described previously (*16*). Active fractions pooled after the ceramic hydroxyapatite column were concentrated using a DEAE-Toyopearl column (1 mL bed volume; approximately 10 mg of protein) and elution with a small volume of 10 mM potassium phosphate (pH 7.0) containing 0.1% DM and 150 mM KCl. These concentrated materials were found to be approximately 95% homogeneous by SDS−7% polyacrylamide gel electrophoresis and were used as the purified wild-type and UQ_8-free forms of mGDH.

Preparation of Holomerized mGDH for Pulse Radiolysis. Both UQ_8-free and UQ_8-bound mGDH were holomerized by incubation with an equimolar concentration of PQQ and 1 mM $MgCl_2$ for 30 min at room temperature. The samples were then immediately frozen in liquid nitrogen for later use in pulse radiolysis experiments. In all cases, this procedure resulted in at least 70% holomerization.

Pulse Radiolysis. Pulse radiolysis experiments were performed using a linear accelerator at The Institute of Scientific and Industrial Research, Osaka University (*23*−*29*). The pulse width and energy were 8 ns and 27 MeV, respectively. The sample was placed in a quartz cell with an optical path length of 1 cm. The temperature of the sample was maintained at 25 °C. The light source for the spectrophotometer was a 200 W Xe lamp. After the light had passed through an optical path, the transmitted light intensities were analyzed and monitored by a fast spectrophotometric system composed of a Nikon monochromator, a photomultiplier (Hamamatsu Photonics, R-928), and a Unisoku data analyzing system. For the time-resolved transient absorption spectral measurement, the monitor light was focused into a quartz optical fiber, which transported the electron pulse-induced transmittance changes to a gated spectrometer (Unisoku, TSP-601-02). The resolution time of spectrophotometer is 10 ns. The concentration of hydrated electrons generated by pulse radiolysis was determined by the absorbance change at 650 nm using an extinction coefficient of 14.1 mM^{-1} cm^{-1} (*32*), and was adjusted by varying the dose of the electron beam.

Samples for pulse radiolysis were prepared as follows. The enzyme solutions, which contained 10 mM potassium phosphate (pH 6−8) and 0.1 M *tert*-butyl alcohol (for scavenging OH radicals), were subjected to repeated deaeration, followed by flushing with Ar gas. The quartz cells had a light path of 1 cm. Although pulse radiolysis did not damage the enzyme, a fresh sample was used for each pulse. Static absorption spectra were recorded with a Hitachi U-3000 spectrophotometer.

RESULTS

Pulse radiolysis results in the instantaneous generation of hydrated electrons (e_{aq}^-), which in turn can reduce redox center(s) within a protein. Pulse radiolysis of the UQ_8-bound form of mGDH causes an increase in the absorbance at 420 nm with a half-time of 2 μs (Figure 1A). The kinetic difference spectrum 10 μs after pulse radiolysis (Figure 2A) shows that the reaction intermediate has an absorption maximum at 420 nm. In the UQ_8-free enzyme, on the other hand, the absorption changes at 420 nm were considerably smaller (Figure 3). This indicates that e_{aq}^- reacts with the UQ_8 bound to the enzyme to form a ubisemiquinone radical. The transient spectrum in Figure 2A is similar to that of the semiquinone neutral radical obtained upon pulse radiolysis of free UQ in aqueous solution (*33*, *34*). On the other hand, the transient spectra of the UQ_8-free enzyme, which has broad absorption below 350 nm, are thought to be due to the reaction of e_{aq}^- with amino acid residues in mGDH (*35*).

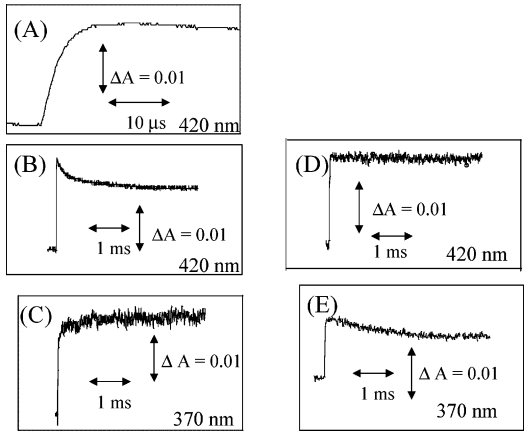

FIGURE 1: Adsorption changes after pulse radiolysis of the UQ_8-bound holoenzyme (A−C) and the UQ_8-bound apoenzyme (D and E). All samples contained 10 mM potassium phosphate buffer (pH 7.4), 0.1 M *tert*-butyl alcohol, and 60.5 μM enzyme.

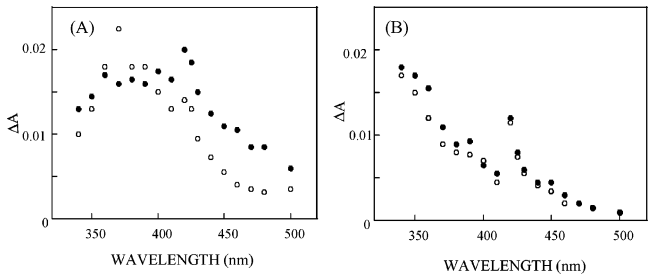

FIGURE 2: Transient absorption spectra 10 (●) and 400 μs (○) after pulse radiolysis of the UQ_8-bound holoenzyme (A) and the UQ_8-bound apoenzyme (B). Pulse radiolysis was carried out as described in the legend of Figure 1.

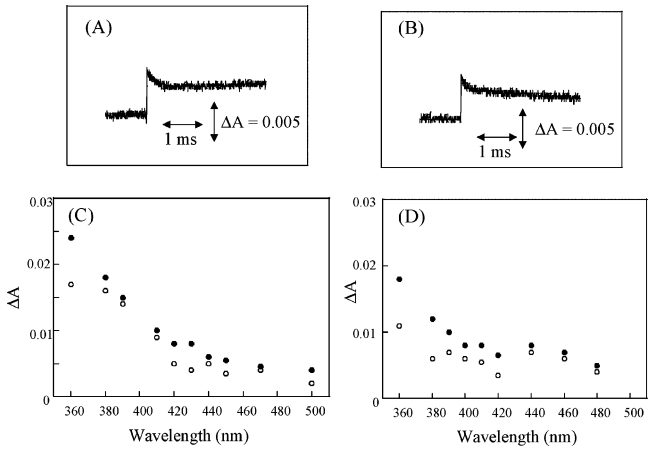

FIGURE 3: Absorption changes at 420 nm after pulse radiolysis of the UQ_8-free holoenzyme (A) and the UQ_8-free apoenzyme (B). Transient absorption spectra 10 (●) and 400 μs (○) after pulse radiolysis of the UQ_8-free holoenzyme (C) and the UQ_8-free apoenzyme (D). All samples contained 10 mM potassium phosphate buffer (pH 7.4), 0.1 M *tert*-butyl alcohol, and 125 μM enzyme.

Subsequently, in the UQ_8-bound holoenzyme, a decay in the absorption at 420 nm was observed on the millisecond time scale (Figure 1B), indicating reoxidation of the ubisemiquinone radical. Concomitantly, an absorbance increase at 370 nm appeared 400 μs after pulse radiolysis (Figures 1C and 2A). As in the case with the holoenzyme, ubisemiquinone was initially formed in the PQQ-free apoenzyme. However, no subsequent absorption changes are observed (Figures 1D,E and 2B). On the basis of these findings, we

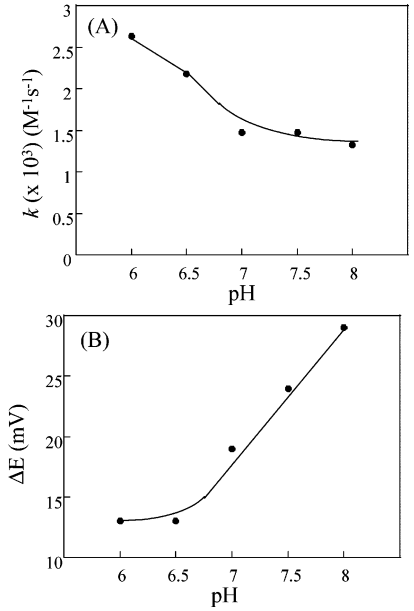

FIGURE 4: (A) pH dependence of the rate constants for intramolecular electron transfer. The rate constants were determined from the recovery of the absorbance at 420 nm. (B) pH dependence of the difference in the redox potential of the PQQ•/PQQ pair minus that of the QH•/Q pair in mGDH. The difference was determined from the ratio of the recovery of the absorbance to the initial increase at 420 nm.

concluded that the UQ_8 bound by mGDH is first reduced and that this is followed by reoxidation of the ubisemiquinone radical and reduction of PQQ.

The reaction of e_{aq}^- with mGDH obeyed pseudo-first-order kinetics, when 0.5−8 μM e_{aq}^- was generated in a solution containing 68 μM mGDH. The second-order rate constant for the reaction of e_{aq}^- with UQ_8 in the enzyme was 5.2×10^{10} M^{-1} s^{-1}. In contrast, the rate constants for the decrease and increase in the absorbance at 420 and 370 nm were 1.2×10^3 s^{-1} at pH 7.0 and were independent of the enzyme concentration (data not shown). Therefore, we concluded that this process is the intramolecular electron transfer from ubisemiquinone to PQQ in mGDH.

Because the pH dependence of the redox properties of UQ has been shown to be important for the function of UQ-binding proteins (*6*, *9*, *10*, *36*, *37*), we next examined the effect of pH on the intramolecular electron transfer. Between pH 6.0 and 8.0, there was no change in the initial transient spectrum for mGDH with an absorption maximum at 420 nm (data not shown). This suggests that the ubisemiquinone bound to mGDH forms the neutral radical in a manner independent of the external pH. The rate constants for the intramolecular electron transfer from ubisemiquinone to PQQ, determined from the absorbance change at 420 nm, decreased slightly as the external pH was increased from 6.0 to 8.0 (Figure 4A). A similar pH dependence of the rate constants was obtained for the intramolecular electron transfer from the ubisemiquinone radical to heme *b* in *E. coli* cytochrome *bo* (*28*).

DISCUSSION

Structure−function studies of the UQ/UQH_2 redox sites in *E. coli* mGDH have revealed the presence and properties of two UQ-binding sites in the enzyme (*22*). One of these sites is for bound UQ (Q_I), and the other is for the bulk UQ

Table 1: Differences in Redox Potential and Intramolecular Electron Transfer Rate Constant for mGDH from the Recovery of the Absorbance at 420 nm

pH	ΔE_0 (mV)	k_1 (s^{-1})	k_{-1} (s^{-1})
6.0	13	1.9×10^3	7.5×10^2
6.5	13	1.1×10^3	4.4×10^2
7.0	19	9.6×10^2	5.1×10^2
7.5	24	9.1×10^2	5.5×10^2
8.0	29	8.0×10^2	5.3×10^2

pool (Q_{II}). Because there is no UQ bound at the Q_{II} site, the data presented here directly demonstrate the involvement of the Q_I site in the intramolecular electron transfer process. Upon pulse radiolysis, the e_{aq}^- transferred one electron to the bound UQ_8 at the Q_I site to yield a ubisemiquinone neutral radical, which, in turn, reduces PQQ on a millisecond time scale. This assignment is supported by the fact that such changes were not observed in the UQ_8-free enzyme or in the PQQ-free apoenzyme. However, the assignment of the formation of the PQQ semiquinone is currently uncertain because there have not been any reports of spectra for the semiquinone form of PQQ in the PQQ-containing dehydrogenase as a single species. The two absorption spectra each for $PQQH_2$ and PQQ semiquinone could not be differentiated during redox titration (*37*, *38*). On the other hand, two types of free PQQ semiquinone have been obtained in aqueous solution by pulse radiolysis (*39*, *40*). The protonated form of the PQQ semiquinone exhibits small absorptions at 480 and 390 nm, whereas the unprotonated form has absorption bands at 460 and below 380 nm. The spectrum of the protonated form is very similar to that observed for the species generated 400 μs after pulse radiolysis (Figure 2A).

Previous reports showed that the electron equilibrates between type I Cu and type II Cu in copper-containing nitrite reductase (*25*) and between copper A and the *a* heme in bovine cytochrome oxidase (*23*) following reductions of the type I copper nitrite reductase or copper A of cytochrome oxidase by pulse radiolysis. These results reflect the relatively small differences between the redox potentials of the pairs of sites in these two enzymes. Similarly, in these experiments, the electron transfer reaction in terms of rapid equilibrium is as follows:

$$UQ\cdots PQQ \xrightarrow[H^+]{e_{aq}^-} {}^{\bullet}UQH\cdots PQQ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} UQ\cdots {}^{\bullet}PQQH$$

This was confirmed by the fact that the absorbance at 420 nm did not return to its initial value. Therefore, the observed first-order rate constants consist of the sum of k_1 (forward) and k_{-1} (backward). Electron transfer in mGDH is normally from PQQ to UQ, and the reverse process presented here is essentially an artificial process, which occurs only under specific conditions. However, the electron transfer is reversible, and the controlled factors are identical in both direction. The difference in the redox potentials (ΔE) of PQQ$^{\bullet}$/PQQ and UQ$^{\bullet}$/UQ pairs can be calculated from the recovery of the absorbance at 420 nm. The k_1, k_{-1}, and ΔE values are presented in Table 1. The redox potential of the PQQ$^{\bullet}$/PQQ pair in the soluble glucose dehydrogenase from *Acinetobacter calcoaceticus* has been determined to be 33 mV by continuous-flow column electrolytic spectroelectrochemistry (*39*). Consequently, in mGDH, the redox potential of the UQ$^{\bullet}$/UQ pair in the bound UQ_8 was estimated to be between 0 and 15 mV, which is similar to the values for bound UQ_8 in cytochrome *bo* (*9*) and cytochrome *bd* (10) (22.5 mV).

The spectra of UQH$^{\bullet}$ (neutral semiquinone radical) and $UQ^{-\bullet}$ (anionic radical) have absorption maxima at 420 and 440 nm, respectively, and a pK_a value of 5.9 when free in solution (*33*, *34*).

$$^{\bullet}UQH \rightleftharpoons {}^{\bullet}UQ^- + H^+$$

UQ-binding proteins, however, form only neutral or anionic semiquinone radicals in a manner independent of the external pH. These data show that the Q_I site in mGDH stabilizes the ubisemiquinone neutral radical, whereas most of the semiquinone radicals observed in EPR spectra were assigned as anionic forms (*8*−*10*, *42*−*44*). Our previous pulse radiolysis experiment for cytochrome *bo* also shows that the anionic form of the ubisemiquinone radical, which exhibits an absorption maximum at 440 nm, is formed (*27*). This suggests that the neutral or anionic radicals are stabilized by the protein to which UQ_8 is bound. The specific interactions are suggested to involve hydrogen bonds from the two carbonyl groups of UQ to the protein, as revealed by a pulsed EPR technique (*42*−*44*).

Interestingly, the interdomain electron transfer from the bound UQ_8 to PQQ (1.2×10^3 s^{-1}) is comparable to the intramolecular electron transfer from type I to type II copper in copper-containing nitrite reductase (1.4×10^3 s^{-1}) (*25*) and from heme *c* to heme d_1 of cytochrome cd_1 nitrite reductase (1.4×10^3 s^{-1}) (*26*). The distance between the two redox centers is 12.5 Å (*45*) for copper-containing nitrite reductase and 11 Å (*46*) for the heme−heme edge distance of cd_1 nitrite reductase. Thus, the distance between UQ_8 and PQQ in mGDH would be 11−13 Å.

The e_{aq}^- is a nonspecific reductant and is very reactive toward amino acid residues of proteins. Thus, when the redox site is not directly exposed to the solvent and is masked by the protein, e_{aq}^- does not react with the redox site but rather with the amino acid residues. A typical example is the reaction of e_{aq}^- with D-amino acid oxidase, a member of the flavoprotein family (*35*). These data showed that the e_{aq}^- did not react with PQQ of mGDH in the absence of UQ_8 (Figure 3C). This is consistent with the proposal that PQQ is buried in a hydrophobic pocket of mGDH (*49*), as suggested by the three-dimensional structure of methanol dehydrogenase (*19*, *48*−*50*). In contrast, the e_{aq}^- effectively reduced the enzyme-bound UQ_8. This result strongly suggests that the Q_I site is located at the surface of the enzyme.

In summary, we report for the first time the transient formation of the ubisemiquinone neutral radical at ambient temperature and the subsequent electron transfer to PQQ in mGDH. The electron equilibrates between the bound UQ_8 at the Q_I site and PQQ. These findings are consistent with our proposal that during the catalytic cycle of the enzyme the bound UQ at the Q_I site mediates electron transfer from the reduced PQQ to UQ at the Q_{II} site.

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