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SULFHYDRYL GROUPS, A FACTOR IN THE POLYMORPHISM OF SUCCINYL-CoA SYNTHETASE (GDP-FORMING)

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SUMMARY

Sulfhydryl reagents and oxidized CoA were examined for their effects on the various forms of pig heart succinyl-CoA synthetase (GDP-forming)(succinate : CoA ligase (GDP-forming), EC 6.2.1.4, also known as succinate thiokinase. Upon treating the polymorphic enzyme with mercaptoethanol or dithiothreitol, the pI 6.2 species was converted to the pI 6.0 form, indicating that the redox states of the enzyme sulfhydryl groups may be responsible for the differences in isoelectric points. Titration of the pI 6.2 and 6.0 enzymes with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) in the presence of sodium dodecylsulfate yielded 35.5 and 38.5 moles of sulfhydryl group per mole of enzyme, respectively. Therefore it appears that the two enzyme forms do not have greatly different sulfhydryl group contents and that only a small number, possibly one or two disulfides are reduced to free sulfhydryls by dithiothreitol in the conversion of the pI 6.2 to pI 6.0 enzyme form.

Incubation of succinyl-CoA synthetase with oxidized CoA before electrofocusing resulted in the formation of two enzyme species with isoelectric points of 5.3 and 5.2; each form had the same amount of bound CoA per unit of enzymic activity. These forms were not observed when dithiothreitol was added to the incubation mixture. Also, the CoA bound to the enzyme was discharged by dithiothreitol, indicating that a disulfide compound is formed from succinyl-CoA synthetase and oxidized CoA. Both the pI 5.3 and 5.2 enzymes contained exchangeable phosphate.

INTRODUCTION

Succinyl-CoA synthetase (GDP-forming) (succinate : CoA ligase (GDP-forming), EC 6.2.1.4, also known as succinate thiokinase) from pig heart has been shown to exist as either a phosphorylated or a non-phosphorylated protein [1]. However, it was recently shown that there are in fact at least five enzyme forms with pI values of

Abbreviation: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

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6.4, 6.2, 6.0, 5.9, and 5.8 [2]. The pI 6.2, 6.0, 5.9 and 5.8 species are all phosphorylated and contain the same number of phosphates bound per unit enzymic activity. The pI 6.4 enzyme is not phosphorylated and has been called the free enzyme form. All of the enzyme species are interconvertible upon the proper treatment and all are similar with respect to K_m for various substrates, molecular weight, pH optimum and heat stability. Also another enzyme form (pI 5.3), which is not a natural constituent of succinyl-CoA synthetase, was seen after incubation of the pI 6.0 enzyme with CoA then GTP.

Since the various enzyme forms are interconvertible, it was concluded that the polymorphism was not caused by genetic variations or other irreversible processes. However, the exact nature of the multiplicity had not been determined. In the present study, we have observed the conversion of the pI 6.2 to the pI 6.0 enzyme form by sulfhydryl reagents and report the existence of a stable disulfide compound formed between the enzyme and oxidized CoA.

MATERIALS AND METHODS

Reagents

Guanine nucleotides and coenzyme A were purchased from P–L Biochemicals, mercaptoethanol, dithiothreitol, sodium dodecylsulfate and 5-5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were obtained from Sigma. [γ-^{32}P]GTP and [8-^{14}C]GTP were obtained from New England Nuclear. Sephadex G-50 was from Pharmacia Fine Chemicals. CoA was tritiated by New England Nuclear [3]. Oxidized CoA and oxidized ^{3}H-labeled CoA were prepared by treating the corresponding reduced compounds with KI_3 then purified by DEAE-bicarbonate column chromatography [3]. Ampholine carrier ampholytes (pH 5–7) were supplied by LKB.

Succinyl-CoA synthetase

The enzyme was purified from pig heart, assayed and electrofocused using an LKB model 8101 column as previously described [2]. The value 8.4 units of succinyl-CoA synthetase per nmole [3] has been used throughout this study as a basis for comparing the relative amounts of ligands bound to the various enzyme forms and for determining the molar concentration of enzyme. The merit of using this value has been discussed [2].

Determination of sulfhydryl groups

The number of sulfhydryl groups per mole of succinyl-CoA synthetase was determined by reaction with DTNB and measuring the absorbance at 410 nm using an ε_M value of 13 700 $M^{-1} \cdot cm^{-1}$ [4, 5]. Incubation mixture contained 0.8 nM succinyl-CoA synthetase, 100 mM Tris–Cl buffer (pH 8.0), 0.25 μM DTNB. When the absorbance at 410 nm became constant, sodium dodecylsulfate (final concentration 1 %) was added to determine the total sulfhydryl content.

RESULTS

Conversion of the pI 6.2 enzyme to the pI 6.0 form

The standard electrofocusing pattern of pig heart succinyl-CoA synthetase is

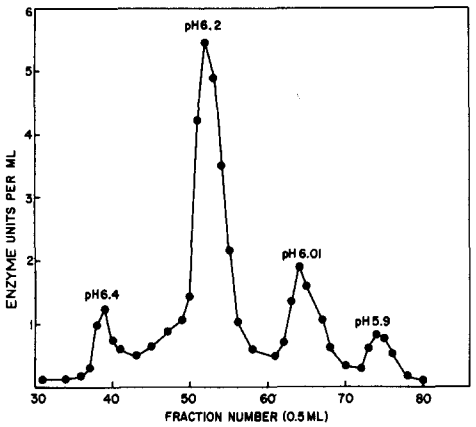

Fig. 1. Electrofocusing succinyl-CoA synthetase. The enzyme (36 units, 80 units/mg), in 100 mM Tris–acetate buffer, pH 7.4, containing 25% glycerol, was added to the electrofocusing column after the sucrose density gradient was formed. Electrofocusing was continued for 45 h with a final power consumption of 0.4 W at 600 V.

illustrated in Fig. 1. There was no detectable pI 5.8 species in this particular preparation. When another aliquot of the same preparation was incubated with 10 mM dithiothreitol for 90 min then electrofocused, it can be seen that the pI 6.2 enzyme was almost completely converted to the pI 6.0 form (Fig. 2). In similar experiments with different enzyme preparations, 1 mM mercaptoethanol gave a qualitatively similar but less dramatic effect. Since dithiothreitol serves as a reducing agent for disulfide groups [6], it appears that the sulfhydryl group oxidation states of the pI 6.2 and 6.0 enzymes may be responsible for the differences in isoelectric points.

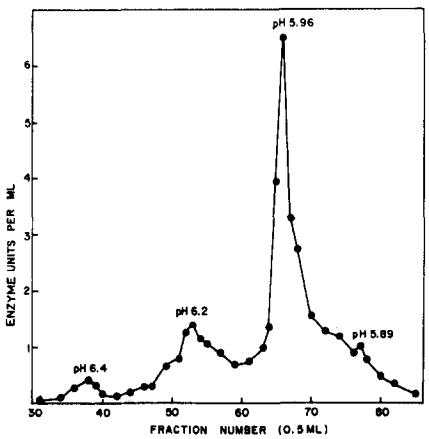

Fig. 2. Electrofocusing succinyl-CoA synthetase after incubation with dithiothreitol. The enzyme (35 units, 80 units/mg) in 100 mM Tris–acetate buffer, pH 7.4, containing 25% glycerol, was incubated for 1.5 h at room temperature with 10 mM dithiothreitol, then electrofocused.

Titration of sulfhydryl groups

In determining the sulfhydryl group content of the pI 6.2 and 6.0 forms, succinyl-CoA synthetase was purified to apparent homogeneity (spec. act. 120) and

electrofocused. Another electrofocusing column (containing ampholine carrier ampholyte, but no enzyme) was prepared and fractions corresponding to the pI 6.2 and 6.0 peaks were used as blanks in the sulfhydryl and protein determinations. The results are shown in Table I. Upon titration with DTNB both the isolated pI 6.2 and 6.0

TABLE I

ACCESSIBLE AND TOTAL SULFHYDRYL GROUPS OF SUCCINYL-CoA SYNTHETASE

The specific activities of the unresolved mixture, the pI 6.2 enzyme and the pI 6.0 enzyme were 103, 121, and 98, respectively. The value $8.4 \cdot 10^9$ units enzyme/mole [2, 3] was used to determine enzyme concentration. The numbers are the average of two determinations.

Enzyme species	Moles of sulfhydryl per mole enzyme	
	Without sodium dodecylsulfate	With sodium dodecylsulfate
Unresolved mixture	10.7	36.6
pI 6.2	11.0	35.5
pI 6.0	12.1	38.5

enzymes were shown to contain 11 and 12.1 moles of sulfhydryl per mole of enzyme, respectively. The enzymes were inactivated by this treatment but greater than 90% of the original activity could be restored by incubation with excess dithiothreitol. When the sulfhydryl titration was performed in the presence of 1% sodium dodecylsulfate, the pI 6.2 and 6.0 enzymes were shown to contain 35.5 and 38.5 moles of sulfhydryl per mole of enzyme, respectively. These data indicate that the two enzyme forms do not have greatly different sulfhydryl group contents and that the conversion of the pI 6.2 and 6.0 enzyme by dithiothreitol may involve the reduction of only a small number of disulfides. The sulfhydryl group titration of an unresolved preparation of purified succinyl-CoA synthetase is also shown in Table I.

Formation of the pI 5.3 and 5.2 enzyme forms

An enzyme species with a pI value of 5.3 had been previously observed [2]. The present study describes an investigation into the formation and nature of this enzyme form. When polymorphic succinyl-CoA synthetase was incubated with GTP, oxidized ^{3}H-labeled CoA and $MgCl_2$, the electrofocusing pattern illustrated in Fig. 3 was seen. Two peaks of enzymic activity, which are not natural constituents of succinyl-CoA synthetase, were present; one at pH 5.3 and the other at pH 5.2. The tritium radioactivity profile indicates that CoA was bound to both enzyme species (approx. 7 nmoles CoA/8.4 units enzyme). The excess unbound ^{3}H-labeled CoA migrated into the anode electrolyte solution and is not illustrated.

Since there is little pI 6.4-free enzyme in these highly purified enzyme preparations (e.g. Fig. 1), the pI 5.3 and 5.2 species must be generated from one or more of the naturally occuring phosphorylated enzyme forms. Therefore, it was of interest to determine whether or not the pI 5.3 and 5.2 enzyme forms had other components of the reaction mixture bound besides ^{3}H-labeled CoA. Incubation of succinyl-CoA synthetase with $MgCl_2$, [8-^{14}C, γ-^{32}P]GTP and oxidized non-labeled CoA before electrofocusing resulted in the pattern illustrated in Fig. 4. Here the pI 5.3 and 5.2

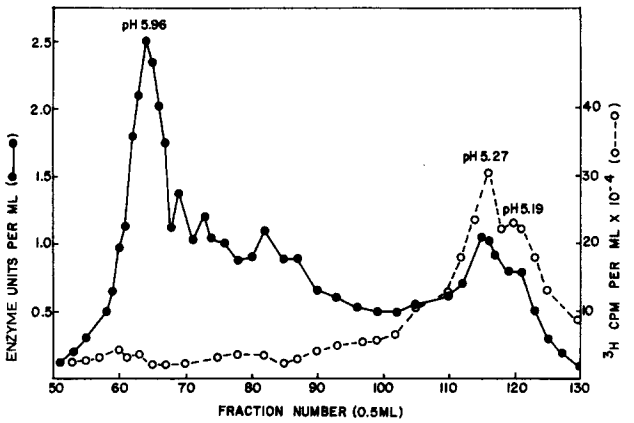

Fig. 3. Isolation of enzyme-bound ^{3}H-labeled CoA by electrofocusing. Succinyl-CoA synthetase was purified to a specific activity of 90 units/mg and dialyzed against 100 mM Tris–acetate buffer (pH 7.4) containing 20% glycerol. The 2.25-ml reaction mixture (in 20% glycerol) contained, in μmoles: oxidized ^{3}H-labeled CoA, 0.15 ($3.3 \cdot 10^8$ cpm/μmole); $MgCl_2$, 2.5; GTP, 0.25; Tris–acetate buffer (pH 7.4) 200; and 82 units of succinyl-CoA synthetase. The mixture was incubated at room temperature for 1.5 h and loaded in the electrofocusing column. The excess unbound ^{3}H-labeled CoA migrated into the anode electrolyte solution, and is not illustrated.

peaks were clearly separated and both contained about 4 nmoles ^{32}P bound per 8.4 enzyme units. Other experiments with non-labeled oxidized CoA have shown that the pI 5.3 and 5.2 forms are generated in the absence of GTP, indicating that phosphate is not discharged from the naturally occuring phosphoenzymes in the formation.

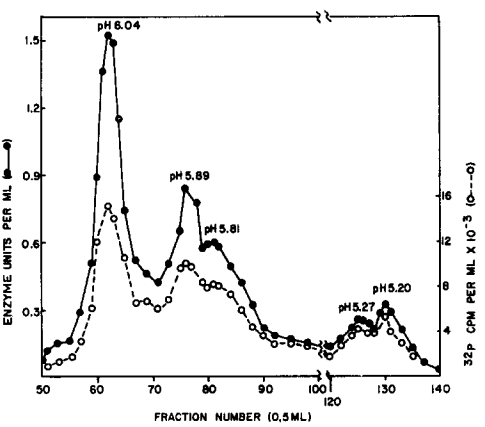

Fig. 4. Demonstration of exchangeable phosphate bound to pI 5.3 and pI 5.2 enzymes. Succinyl-CoA synthetase was purified to a specific activity of 94 units/mg and dialyzed against 100 mM Tris–acetate buffer (pH 7.4) containing 15% glycerol. The 1.35-ml reaction mixture (in 15% glycerol) contained, in μmoles: oxidized non-labeled CoA, 0.25; $MgCl_2$, 1.5; [8-^{14}C,γ-^{32}P]GTP, 0.3 ($1.2 \cdot 10^7$ cpm of ^{14}C, $1.5 \cdot 10^7$ cpm of ^{32}P, respectively); Tris–acetate buffer (pH 7.4) 100; and 29 units of succinyl CoA synthetase. The mixture was incubated at room temperature for 1.5 h and loaded in the electrofocusing column. The ^{14}C radioactivity, which was not above background, and the excess unbound ^{14}C and ^{32}P radioactivity, which migrated into the anode electrolyte solution, are not illustrated.

Effects of dithiothreitol on the enzyme–CoA compounds

Since the pI 5.3 and 5.2 enzymes were seen after succinyl-CoA synthetase was incubated with oxidized CoA, it was thought that enzyme–CoA disulfide compounds were being formed. If this were so, then the addition of dithiothreitol to a reaction mixture containing succinyl-CoA synthetase and oxidized CoA should reduce the enzyme–CoA disulfide complex and discharge the bound CoA. This was observed in an experiment not illustrated, and indeed upon electrofocusing neither the pI 5.3 nor the pI 5.2 enzymes were present.

The discharge of CoA from the enzyme–CoA compound by dithiothreitol was also seen in another series of experiments. It has been previously postulated that

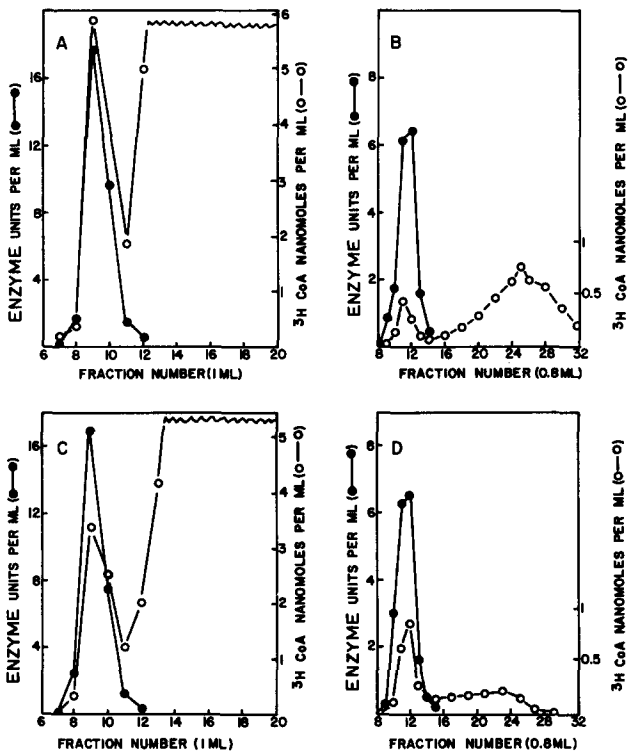

Fig. 5. Effects of dithiothreitol on the enzyme–CoA compounds. In Experiment A, the enzyme–CoA compound was prepared by mixing succinyl-CoA synthetase with in μmoles, in 0.66 ml of 15% glycerol) oxidized ^{3}H-labeled CoA, 0.15 ($3.3 \cdot 10^8$ cpm/μmole); GTP, 0.1; $MgCl_2$, 5; Tris–acetate buffer (pH 8) 50; and 41 units of succinyl-CoA synthetase (spec. act. 80). The mixture was incubated for 1 h at room temperature and filtered through a Sephadex G-50 column. In Experiment B, the enzyme from the peak tube in Experiment A (17 units, 3 nmoles ^{3}H-labeled CoA/8.4 units enzyme) was incubated for 45 min with 5.3 mM dithiothreitol then applied to another Sephadex G-50 column. In experiment C, the enzyme–CoA complex was prepared by incubating succinyl-CoA synthetase with reduced ^{3}H-labeled CoA. The 0.68-ml reaction mixture (in 15% glycerol) contained, in μmoles: reduced ^{3}H-labeled CoA 0.15 ($3.3 \cdot 10^8$ cpm/μmole); $MgCl_2$, 5; Tris–acetate buffer (pH 8) 50; and 41 units of succinyl-CoA synthetase (spec. act. 80). The mixture was incubated for 1 h at room temperature and filtered through a Sephadex G-50 column. In Experiment D, the enzyme from the peak tube in Experiment C (17 units, 1.5 nmoles ^{3}H-labeled CoA/8.4 units enzyme) was incubated for 45 min with 5.3 mM dithiothreitol. In each of the above experiments the Sephadex G-50 columns (1.2 cm × 20 cm) were eluted with 100 mM Tris–acetate buffer (pH 8) containing 20% glycerol.

reduced ^{3}H-labeled CoA reacts with succinyl-CoA synthetase to form a high energy E–CoA complex [1]. However, if this high energy enzyme form occurs, it is apparently too unstable to be detected after electrofocusing for 45 h [2]. Nevertheless, it is interesting to use gel filtration to compare the effect of dithiothreitol on the enzyme–CoA complexes formed between succinyl-CoA synthetase and either oxidized or reduced ^{3}H-labeled CoA. In Experiment A (Fig. 5), succinyl-CoA synthetase was incubated with oxidized ^{3}H-labeled CoA and filtered through a Sephadex G-50 column; the succinyl-CoA synthetase in the peak tube contained 3 nmoles CoA per 8.4 units enzyme. When the enzyme from this peak tube was incubated with dithiothreitol and applied to another Sephadex G-50 column (Experiment B), it was seen that the reducing agent discharged 82% of the bound ^{3}H-labeled CoA. Similarly succinyl-CoA synthetase was incubated with reduced ^{3}H labeled CoA (Experiment C). The enzyme in the peak tube bound 1.5 nmoles CoA per 8.4 units of succinyl-CoA synthetase, and only 33% of the bound ^{3}H-labeled CoA was discharged by dithiothreitol (Experiment D). It is not surprising that the enzyme bound only 3 nmoles CoA per 8.4 units in the Sephadex experiments with oxidized ^{3}H-labeled CoA whereas 7 nmoles CoA were bound per 8.4 units in Fig. 3. This is simply because in the former case the pI 5.3 and 5.2 enzymes were not separated from the remaining unreacted phosphoenzymes; in the latter case they were.

DISCUSSION

The technique of isoelectric focusing has been used to examine the effect of sulfhydryl reagents on the various forms of pig heart succinyl-CoA synthetase. The marked conversion of the pI 6.2 to the pI 6.0 enzyme by disulfide-reducing agents (mercaptoethanol and dithiothreitol) indicates that the redox states of the enzyme sulfhydryl groups are responsible at least in part for the differences in isoelectric points.

Since titration of the pI 6.2 and 6.0 forms by DTNB shows that the two enzymes do not differ greatly in their total sulfhydryl group contents (35.5 and 38.5 moles/mole of enzyme, respectively), it is possible that only one or two disulfides are involved in the interconversion of the pI 6.2 and 6.0 enzymes. This would be consistent with a requirement that the sulfhydryl groups involved must be in close proximity in order to react with one another. The decrease in isoelectric point may be caused by the addition of two or four acidic sulfhydryl groups, the resulting conformational change, or both.

There are more than two different states of the phosphoenzyme, yet only one free enzyme has been observed upon electrofocusing [2]. These two facts would not contradict each other if the free enzyme exists in various oxidation states having the same isoelectric point or if the free enzyme form of the pI 6.0 enzyme is auto-oxidized to the free form of the pI 6.2 enzyme. Evidence that the latter may occur was obtained when phosphorylation of the pI 6.4-free enzyme resulted in the formation of predominantly the pI 6.2 phosphoenzyme [2].

All data obtained in this laboratory [2] indicate that the two major phosphorylated enzymes (pI 6.2 and 6.0) do not qualify as isoenzymes. They have identical physical and kinetic properties and they are interconvertible by dithiothreitol and mercaptoethanol. Similar sulfhydryl reagent induced interconversions have been ob-

served with the multiple forms of phosphoglucose isomerase [7], dihydrofolate reductase [8] and glucose-6-phosphate dehydrogenase [9, 10]. In fact, multiplicity caused by different states of sulfhydryl group oxidation may prove to be an important factor in explaining the microheterogeneity observed upon electrofocusing a variety of presumably homogenous proteins [11].

Upon incubating succinyl-CoA synthetase with oxidized CoA, we have observed the formation of two species (pI 5.3 and pI 5.2) which are not natural constituents of the enzyme. The decrease in isoelectric point (up to 0.9 pH unit) is most likely caused by the large number of acidic functions introduced by binding 7 nmoles CoA per 8.4 units of enzyme. Most of the enzyme sulfhydryl groups accessible to DTNB (11–12.1 moles/mole enzyme) may be also capable of reacting with oxidized CoA. Phosphate is not discharged from the pI 6.2 and 6.0 phosphoenzymes in the formation of the pI 5.3 and 5.2 enzyme–CoA compounds. Also, neither the pI 5.3 nor the pI 5.2 species are observed when dithiothreitol is added to the incubation mixture containing phosphoenzyme and oxidized CoA. The above data indicate that the pI 5.3 and 5.2 species are mixed disulfide compounds formed between the naturally occuring phosphorylated succinyl-CoA synthetase and CoA. It is not known, however, why they differ in isoelectric point since both forms contain the same number of bound CoA and phosphate per enzyme unit.

We have also examined the effect of dithiothreitol on the enzyme–CoA compound formed from succinyl-CoA synthetase and oxidized CoA and the effect of dithiothreitol on an unstable complex formed from the enzyme and reduced CoA. This latter complex most likely involves interactions at the active site, since electrofocusing the enzyme which had been incubated with reduced CoA has shown that phosphate was discharged from the pI 6.2 and 6.0 phosphoenzymes and that the non-phosphorylated pI 6.4-free enzyme was formed [2]. But for this study, it is immaterial whether it is a high energy complex or simply reflects the high affinity of reduced CoA for the active site. In fact work in another laboratory [12] has shown that a high energy E–CoA complex probably does not exist. However it is significant that after treatment with dithiothreitol only 33% of the CoA was discharged from the species formed from succinyl-CoA synthetase and reduced CoA, whereas dithiothreitol discharged greater than 80% of the CoA from the compound formed from succinyl-CoA synthetase and oxidized CoA. This shows that the two enzyme–CoA complexes are different and supports the hypothesis that the pI 5.3 and pI 5.2 species are actually mixed disulfides. It is also noteworthy that in the experiments with reduced CoA, 1.5 and 1.0 nmoles of CoA were bound per 8.4 units of enzyme after the first and second gel filtrations, respectively. These numbers are significantly higher than that of 0.1–0.8 nmole per 8.4 units observed previously in experiments in which glycerol was not included [3]. Perhaps glycerol has a stabilizing effect on the complex formed between the enzyme and reduced CoA.

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