The phosphonomethyl analogue of 3-phosphoglycerate is a potent competitive inhibitor of phosphoglycerate mutases

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The phosphonomethyl analogue of 3-phosphoglycerate (2-hydroxy-4-phosphonobutanoate) is a potent competitive inhibitor of cofactor-dependent phosphoglycerate mutase from yeast and of cofactor-independent phosphoglycerate mutase from wheat germ. For the yeast enzyme K_i is 1.3 mM (K_m for substrate is 0.71 mM); for the wheat-germ enzyme K_i is 18 mM (K_m for substrate is 0.86 mM). This analogue should be a useful tool for n.m.r. spectroscopic studies on the mechanism of action of the two mutases. The arsonomethyl analogue of 3-phosphoglycerate (4-arsono-2-hydroxybutanoate) was a relatively poor inhibitor.

Phosphoglycerate mutase (EC 5.4.2.1) is the glycolytic enzyme that catalyses the interconversion of 2-phosphoglycerate and 3-phosphoglycerate. There are two distinct types of enzyme. One type, which is isolated from yeast (*Saccharomyces cerevisiae*), mammals and many other sources, is dependent on the cofactor 2,3-bisphosphoglycerate for activity. In contrast, the other type of enzyme, isolated from certain higher plants, fungi, bacteria and insects, is active in the absence of the cofactor. The present study was initiated by a desire to find substrate analogues that would bind well to the active sites of the two types of phosphoglycerate mutase, but not take part in the reactions. We are particularly interested in compounds that may be suitable for n.m.r.-spectroscopic studies to elucidate the role of the C-terminal ten residues of the enzymes. These residues constitute a flexible tail in yeast phosphoglycerate mutase, and are essential for activity (Chiba *et al.*, 1970; Winn *et al.*, 1981).

The analogues of 3-phosphoglycerate used in the present study are isosteric with the natural substrate, and involve only the replacement of $-O-PO_3H_2$ either by $-CH_2-PO_3H_2$ or by $-CH_2-AsO_3H_2$. These compounds have previously been shown to be substrates of phosphoglycerate kinase (Dixon & Sparkes, 1974; Orr & Knowles, 1974; Adams *et al.*, 1983). The effect of 3-arsono-2-(arsonomethyl)propanoic acid, $(H_2O_3As-CH_2-)_2CH-CO_2H$, was also investigated, although this compound is not an isosteric analogue of the natural substrates and effectors of these enzymes; nevertheless parts of its molecule

are isosteric with 2-phosphoglycerate and 2,3-bisphosphoglycerate, and also with 2-phosphoglycollate, which is an inhibitor of yeast phosphoglycerate mutase, as reviewed by Rose (1980).

Experimental

Substrates and analogues

D-3-Phosphoglycerate, disodium salt (grade 1), and D-2-phosphoglycerate, sodium salt, were from Sigma Chemical Co. (Kingston-upon-Thames, Surrey, U.K.).

D-2,3-Bisphosphoglycerate was from Boehringer (Mannheim, Germany). The 3-phosphonomethyl analogue of 3-phosphoglycerate, i.e. DL-2-hydroxy-4-phosphonobutanoic acid,

and the 3-arsonomethyl analogue of 3-phosphoglycerate, i.e. DL-4-arsono-2-hydroxybutanoic acid,

were synthesized as described previously (Dixon & Sparkes, 1974; Adams *et al.*, 1983). The DL-3-arsono-2-(arsonomethyl)propanoic acid,

was prepared from 3-bromo-2-(bromomethyl)propanoic acid, $(Br-CH_2-)_2CH-CO_2H$, which had been made from diethyl malonate by treatment with formaldehyde and HBr (Ferris, 1955; Stubbe & Kenyon, 1972). This acid (8.3 g) was added in

portions to a hot solution of 10g of NaOH and 5g of As_2O_3 in 10ml of water, and was incubated at 105°C for 3h. The solution was diluted with water, and passed through a column of the H^+ form of a sulphonic resin (Duolite 225) to remove Na^+ ions, and was evaporated to dryness. The resultant syrup was dissolved in water, adjusted to pH6 with cyclohexylamine, and evaporated to dryness again. The white solid was largely dissolved in hot 96% (v/v) ethanol, and the solution was filtered. Diethyl ether was added to the filtrate, and the product crystallized. It proved to be homogeneous on paper electrophoresis at pH 3.5 (methods of Adams *et al.*, 1983). On conversion into the free acid by repetition of the treatment with resin, and evaporation to dryness, a crystalline sample was obtained that gave an analysis of: C, 14.1%; H, 3.1% ($\text{C}_4\text{H}_{10}\text{As}_2\text{O}_8$ requires C, 14.3%; H, 3.0%).

Yeast phosphoglycerate mutase

Cofactor-dependent phosphoglycerate mutase was prepared from dried baker's yeast (*Saccharomyces cerevisiae*) (Distillers Co.) by a modification of the procedures described by de la Morena *et al.* (1968) and Price & Jaenicke (1982). Dried yeast (50g) was extracted overnight (16–18h) at room temperature ($15\text{--}20^\circ\text{C}$) with 100ml of 1M- NH_3 in a 500ml conical flask. $(\text{NH}_4)_2\text{SO}_4$ (70g), 6ml of 0.5M- Na_4EDTA and 223ml of distilled water heated to about 90°C were added, and the temperature quickly brought to 70°C in a boiling-water bath. This temperature, $\pm 1^\circ\text{C}$, was maintained for 5min, the mixture was then rapidly cooled to $0\text{--}4^\circ\text{C}$ in a propan-2-ol/solid- CO_2 bath. All subsequent operations were done at 4°C or in an ice bath. Insoluble material was removed by centrifugation at $9000g$ for 15min, and protein in the supernatant (fraction 1) was collected by $(\text{NH}_4)_2\text{SO}_4$ precipitation (30g was added, with stirring, to each 100ml of supernatant). After 5min the precipitate was collected by centrifugation at $7000g$ for 10min, and was then dissolved in water to give a volume of about 100ml (fraction 2). This fraction may be stored overnight at 4°C with negligible loss of mutase activity.

Acetone precipitation was done in an ice bath. One volume of acetone, precooled to about -20°C , was added to fraction 2 with stirring. After 5min the precipitate was collected by centrifugation at $4000g$ for 10min and then extracted twice with water with the aid of a glass homogenizer. Centrifugation in each case was at $7000g$ for 10min. The volume of the combined extracts (fraction 3) was about 50ml. This was fractionated with $(\text{NH}_4)_2\text{SO}_4$, and the protein precipitated in the range 55–65% saturation was collected, dissolved in a minimum volume of 10mM-Tris/HCl

buffer, pH8.0, and dialysed overnight at 4°C against two changes of the same buffer (fraction 4).

Fraction 4 was applied to a DEAE-cellulose (Whatman DE-52) column (1.5cm $\times$ 30cm) equilibrated with the same Tris/HCl buffer. The column was washed (flow rate 60ml/h) until all unbound protein was removed. The cofactor-dependent mutase was eluted with a linear NaCl gradient (300ml of 10mM-Tris/HCl buffer, pH8.0, and 300ml of 0.2M-NaCl in the same buffer). Active fractions were pooled. Protein concentrations were measured by the method of Lowry *et al.* (1951), with bovine albumin as standard. A specific activity of about 10000 units/mg of protein was usually obtained. Occasionally sodium dodecyl sulphate/polyacrylamide-gel electrophoresis indicated the presence of small amounts of contaminating proteins, which were removed by gel filtration on Sephacryl S-200 (Pharmacia) [1.5cm $\times$ 100cm column eluted with 1% (w/v) NH_4HCO_3 at 25ml/h]. The purified mutase was stored as a suspension in 70%-saturated $(\text{NH}_4)_2\text{SO}_4$ at 4°C . Under these conditions the enzyme was stable for at least a year. The purified yeast phosphoglycerate mutase can lose activity on freeze-drying.

Wheat-germ phosphoglycerate mutase

A modification of the method of Britton *et al.* (1971) was used to purify cofactor-independent phosphoglycerate mutase. The final material was eluted as a single symmetrical peak from Sephacryl S-200, but sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed three minor bands of lower M_r in addition to the main band of mutase (M_r about 60000). The material with lower M_r appears to be derived from the mutase, as the relative intensities of the lower bands increase when enzyme preparations are stored or subjected to further gel filtration (S. M. McAleese, unpublished work). The cofactor-independent mutase used in the present studies has a specific activity of 4000 units/mg.

Measurement of enzyme activity

The cofactor-dependent mutase was assayed by the enolase-coupled assay described by Grisolia (1962), with a total assay volume of 1ml. Routinely, a solution (940 μl) containing the following reagents was equilibrated at 30°C : 50 μl of 200mM-3-phosphoglycerate containing 6mM-2,3-bisphosphoglycerate, 50 μl of 1M-Tris/HCl buffer, pH7.0, and 50 μl of 100mM- MgSO_4 . The reaction was started by the addition of 10 μl of enolase (10 units) (type III from yeast; Sigma Chemical Co.) and 50 μl of suitably diluted mutase. Enzyme solutions were diluted for assay with 0.2% (w/v) bovine albumin (fraction V; Sigma Chemical Co.) in

water. The formation of phosphoenolpyruvate was followed by an increase in absorbance at 240 nm. The rate was calculated over 2 min. An increase in absorbance of 0.1/min corresponds to 1 unit. We prefer to define enzyme activity in these terms rather than in katal, because it is known that there is not a stoichiometric conversion of 2-phosphoglycerate into phosphoenolpyruvate under the conditions of assay (Grisolia & Cleland, 1968). As an approximation, 1 unit corresponds to about 1.4 nmol of 3-phosphoglycerate converted/s. When appropriate, substrate analogues were included in the solution equilibrated at 30°C before the addition of enzymes.

The cofactor-independent mutase was assayed in the same manner except that the 2,3-bisphosphoglycerate was omitted, and the buffer was 50 mM-Tris/HCl, pH 8.7.

Results

Determination of K_m for 3-phosphoglycerate

The effect of substrate concentration on the activities of cofactor-dependent and cofactor-independent mutases is shown in Fig. 1(a). As has been noted before for the cofactor-dependent mutase (see, e.g., Chiba *et al.*, 1970), the enzymes show inhibition at high concentrations of substrate. A substrate concentration of 10 mM was therefore used for routine assay of both enzymes, although earlier workers have used 20 mM. In the

case of the dependent mutase, the cofactor 2,3-bisphosphoglycerate was added to the substrate in a molar ratio of 3%. At high substrate concentrations there was a noticeable lag before a linear rate of reaction was attained. This lag could be abolished by preincubation of the enzyme with the 2,3-bisphosphoglycerate. Grisolia & Cleland (1968) also describe an 'induction period' in assays with high substrate concentration, which could be shortened by increasing the concentration of cofactor. Chiba *et al.* (1970) established that, although 2,3-bisphosphoglycerate is essential for activity, in excess it acted as a competitive inhibitor.

The velocity data for both enzymes, plotted by the method of Lineweaver & Burk (1934), are shown in Fig. 1(b). The value of K_m for 3-phosphoglycerate is 0.71 ± 0.05 mM for the cofactor-dependent mutase and 0.86 ± 0.04 mM for the cofactor-independent mutase. A value of 0.6 mM was obtained by Chiba *et al.* (1970) with the dependent mutase under similar assay conditions but with quantification of the phosphate in phosphoenolpyruvate. Differences from other published values for K_m (0.1–0.3 mM) (see Winn *et al.*, 1981) probably reflect differences in assay conditions. There appears to be some variation between preparations of wheat-germ cofactor-independent mutase. A sample of enzyme purified subsequently to the work reported here had a K_m value for 3-phosphoglycerate of 0.51 ± 0.02 mM.

Fig. 1. Dependence of mutase velocity on substrate concentration

Cofactor-dependent mutase (●) from yeast and cofactor-independent mutase (■) from wheat germ were assayed at various concentrations of 3-phosphoglycerate as substrate as described in the text. (a) Mutase velocity is expressed as a percentage of the highest value obtained. The enzyme concentration was such that this value was 0.14 unit for the dependent mutase, and 0.30 unit for the independent. (b) The results for the dependent mutase were from four separate experiments, and those for the independent mutase from three. The lines were fitted by use of the microcomputer program GENFIT (Powell, 1970). In each experiment the amount of enzyme used gave a velocity of about 0.02 unit in 5 mM substrate, and all rates are expressed relative to this value, which was taken as unity.

Values of 0.6 mM (Ito & Grisolia, 1959) and 0.33 mM (Britton *et al.*, 1971) have also been reported.

Although activity of the independent mutase is not dependent on 2,3-bisphosphoglycerate, the effect of different concentrations of this cofactor were investigated, to see if it would act as a competitive inhibitor of the enzyme. A mixed result was obtained. In the presence of low concentrations of 2,3-bisphosphoglycerate (1 or 2 mM) there was a small increase in mutase activity. This ranged from about 5% with 5 mM-3-phosphoglycerate to about 15% with 0.2 mM-3-phosphoglycerate. Higher concentrations of 2,3-bisphosphoglycerate (5 or 10 mM) inhibited mutase activity. With 10 mM-2,3-bisphosphoglycerate there was 30% inhibition of the mutase assayed with 5 mM-3-phosphoglycerate, and over 60% inhibition if the substrate concentration was only 0.2 mM. No formation of phosphoenolpyruvate could be shown if 2,3-bisphosphoglycerate replaced 3-phosphoglycerate in the assay at a concentration of 1 or 2 mM, and so it appears to be unable to donate a phospho group to the enzyme and thus release 2-phosphoglycerate or 3-phosphoglycerate. This is consistent with the finding by Breathnach & Knowles (1977) that wheat-germ phosphoglycerate mutase preparations showed no phosphatase activity towards 2,3-bisphosphoglycerate.

Effect of substrate analogues

The effects of three substrate analogues on the activities of the cofactor-dependent and cofactor-independent mutases were investigated. As phosphoglycerate mutase activity is measured by a coupled assay with enolase, the effects of these compounds on the activity of enolase was checked. Enolase was assayed under the same conditions as those used for both mutases with 2-phosphoglycerate as substrate. All substrate analogues were racemic mixtures and were assumed to have equal amounts of the D- and L-isomers. The concentrations of analogues used in the present study refer to the concentration of the D-isomer only, as it is known that phosphoglycerates with L-configuration are very poor ligands (Pizer & Ballou, 1959).

Preliminary studies showed that the phosphonomethyl analogue of 3-phosphoglycerate was an inhibitor of both mutases at concentrations equivalent to the substrate concentration. It had no effect on enolase assayed at pH 7, and caused a slight activation of that enzyme assayed at pH 8.7. The arsonomethyl analogue of 3-phosphoglycerate had negligible effect on either mutase at the same concentration as the substrate, but did act as an inhibitor when present in 10-fold molar excess. The 3-arsono-2-(arsonomethyl)propanoic acid inhibited both enzymes when present at the same

concentration as the substrate. At this concentration (10 mM) it also caused inhibition of enolase at pH 7, although this could be overcome by increasing the amount of enolase added to the assay. There was no inhibition of enolase at pH 8.7.

The inhibition by the phosphonomethyl analogue of 3-phosphoglycerate was investigated in more detail.

Inhibition by the phosphonomethyl analogue of 3-phosphoglycerate

Double-reciprocal plots for both enzymes at different inhibitor concentrations are shown in Fig. 2. These data were replotted (Figs. 3 and 4) as described by Cornish-Bowden (1979) to distinguish between the competitive and uncompetitive components of the inhibition, and to obtain values of K_i (the competitive component) and (if appropriate) K'_i (the uncompetitive component).

The results for the wheat-germ cofactor-independent mutase (Fig. 3a) are a good example of competitive inhibition. The point of intersection of the lines gives a value of 18 mM for K_i . The data were replotted as shown in Fig. 3(b) to determine if the inhibition is purely competitive or if there is a significant value for K'_i . The inhibition is largely competitive, as the value for K'_i calculated from the point of intersection of the lines would be 200 mM.

The corresponding results for the yeast cofactor-dependent mutase are not quite so straightforward, presumably because of the presence of the cofactor 2,3-bisphosphoglycerate, which would be competing for the same binding site. The value of K_i (Fig. 4a) is between 1.2 and 1.4 mM. The inhibition is probably largely competitive, as indicated in Fig. 4(b), although the results are less clear-cut than for the cofactor-independent mutase.

Discussion

Yeast cofactor-dependent phosphoglycerate mutase has been particularly well characterized. The enzyme is a tetramer with identical subunits of M_r 26 882. The amino acid sequence (Fothergill & Harkins, 1982) and the 0.28 nm-resolution crystallographic structure (Winn *et al.*, 1981) have been determined. The polypeptide backbone folds into a single domain with a core of six β -strands surrounded by α -helices in an arrangement reminiscent of that found in many glycolytic enzymes and other $\alpha\beta$ -proteins. The most prominent features of the active site are two histidine side chains, which are parallel and about 0.4 nm apart. His-8 is at the C-terminal end of strand 1 of the β -sheet, and His-179 is at the C-terminal end of strand 4. His-8 is phosphorylated during catalysis, and it is likely that His-179 acts as the proton donor/acceptor

Fig. 2. Inhibition of phosphoglycerate mutases by the phosphonomethyl analogue of 3-phosphoglycerate. Cofactor-independent mutase from wheat germ (a) and cofactor-dependent mutase from yeast (b) were assayed in the presence of different concentrations of DL-2-hydroxy-4-phosphonobutanoate as described in the text. The inhibitor concentration values refer to the concentration of the D-isomer. ●, No inhibitor; ■, 1 mM inhibitor; ▲, 2 mM inhibitor; ○, 5 mM inhibitor; □, 10 mM inhibitor. Details are as given in the legend to Fig. 1(b).

(Rose, 1980). The C-terminal ten residues of the mutase are not observed in the electron-density map, presumably because they constitute a flexible tail in the enzyme that was crystallized in the absence of ligands. The residues are of considerable importance, as they are required for activity (Chiba *et al.*, 1970; Winn *et al.*, 1981), and it is likely that they modulate access to the active site, thus excluding water and ensuring that the phospho group is transferred from the enzyme to the substrate and not to water.

A proposed reaction sequence (Fothergill-Gilmore, 1985) based on structural and kinetic studies is shown in Fig. 5. The priming of the reaction occurs when 2,3-bisphosphoglycerate binds in one of two possible orientations to the unligated form of the enzyme shown in the centre of the diagram. Phospho transfer to His-8 would then occur, with His-179 donating the proton required in the reaction. The 3-phosphoglycerate or 2-phosphoglycerate thus formed would then be released, leaving the enzyme in its catalytically competent, phosphorylated, state (diagram e). A round of catalysis involving a 2,3-bisphosphoglycerate intermediate would be initiated when either 3-phosphoglycerate (for glycolysis) or 2-phosphoglycerate (for gluconeogenesis) was bound to the active site. Note that

the 2,3-bisphosphoglycerate complex is the same as the initial 'primed' complex, and that there is a requirement for the 2,3-bisphosphoglycerate to reorientate during each round of catalysis. It seems likely that the C-terminal tail is important for retaining the 2,3-bisphosphoglycerate near the active site during reorientation. The relative concentrations of 3-phosphoglycerate and 2-phosphoglycerate would presumably influence whether the mutase acted glycolytically or gluconeogenically.

Cofactor-independent phosphoglycerate mutase is less well characterized structurally, although many aspects of its activity have been studied. The reaction catalysed by the wheat-germ cofactor-independent mutase has been shown to be intramolecular, and probably involves two phospho transfers via a transient phosphoenzyme intermediate (Britton *et al.*, 1971; Gatehouse & Knowles, 1977; Blättler & Knowles, 1980). In contrast with the cofactor-dependent mutase, however, it has not yet proved possible to isolate the phosphorylated form of the cofactor-independent enzyme.

A number of polycarboxylic and polyphospho compounds such as inositol hexakisphosphate have been shown to be strong competitive inhibi-

Fig. 3. Determination of K_i for the phosphonomethyl analogue of 3-phosphoglycerate with wheat-germ cofactor-independent mutase

The data shown in Fig. 2(a) were replotted. The inhibitor concentrations refer to the D-isomer. (a) Plot of $1/\text{velocity}$ against inhibitor concentration at various 3-phosphoglycerate concentrations. (b) Plot of 3-phosphoglycerate concentration/velocity against inhibitor concentration at various 3-phosphoglycerate concentrations. ●, 5 mM substrate; ■, 2 mM substrate; ▲, 1 mM substrate; ○, 0.5 mM substrate; □, 0.2 mM substrate. Lines were fitted by linear-regression analysis.

Fig. 4. Determination of K_i for the phosphonomethyl analogue of 3-phosphoglycerate with yeast cofactor-dependent mutase. The data shown in Fig. 2(b) were plotted as described in the legend to Fig. 3.

tors of the cofactor-dependent mutase (reviewed by Rose, 1980). These compounds probably interact with the numerous basic residues surrounding the active site, but are unlikely to entice the C-terminal tail to adopt its ligand-bound conformation. The isosteric phosphonomethyl and arsono-

methyl analogues of 3-phosphoglycerate, however, appear to be much more suitable candidates for study of the role of the tail. Previous work with a number of phosphonomethyl analogues has shown them to be good substrates for fructose biphosphate aldolase, phosphoglycerate kinase, glyceral-

Fig. 5. Proposed reaction sequence of cofactor-dependent phosphoglycerate mutase

The drawings represent the active site of the enzyme with the vertical lines corresponding to His-8 (on the left) and His-179. The numerals 2 and 3 label C-2 and C-3 of glycerate. Symbols (IUPAC-IUB Commission on Biochemical Nomenclature, 1978): Gri-2,3- P_2 , 2,3-bisphosphoglycerate; Gri-2- P , 2-phosphoglycerate; Gri-3- P , 3-phosphoglycerate; P , $-PO_3H_2$ and its ionized forms. We presume that the phosphonomethyl analogue of 3-phosphoglycerate will bind to the active site in a manner similar to the natural substrates shown in diagrams a, b, and c, but is unable to undergo the phosphotransfer reaction to produce 2-phosphoglycerate as shown in diagram d.

dehyde-3-phosphate dehydrogenase, glycerol-3-phosphate dehydrogenase, glucose-6-phosphate isomerase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Dixon & Sparkes, 1974; Stribling, 1974; Orr & Knowles, 1974; Adams *et al.*, 1974; Webster *et al.*, 1976). None of these reactions would involve the transfer of the $-CH_2PO_3H_2$ group that had replaced an $-OPO_3H_2$ group. The 1-phosphonomethyl analogue of fructose 1,6-bisphosphate, however, was a good inhibitor of fructose biphosphatase (K_i 70 μM ; K_m for substrate 4 μM), as in this case the enzyme was unable to catalyse the cleavage of the phosphonomethyl moiety (Stribling, 1974).

Our results show that the phosphonomethyl analogue of 3-phosphoglycerate is a potent competitive inhibitor of the cofactor-dependent mutase (K_i 1.3 mM; K_m for substrate 0.71 mM), and a good although less strong inhibitor of the cofactor-independent mutase (K_i 18 mM; K_m for substrate 0.86 mM). We presume that the phosphonomethyl analogue binds to the enzyme in the same manner as the substrate, but is unable to undergo transfer of the 3-phosphonomethyl group (see Fig. 5). The

other two analogues tested are less satisfactory for our further studies to compare and contrast the activities of these enzymes. The arsonomethyl analogue of 3-phosphoglycerate has a lower affinity for the enzymes, and the 3-arsono-2-(arsono-methyl)propanoic acid was an inhibitor of enolase in the coupled assay.

The difference in susceptibility of the two enzymes to inhibition by the phosphonomethyl analogue of 3-phosphoglycerate is of interest. This difference becomes all the more marked when the pH values of the assays are taken into account. The cofactor-dependent enzyme, which is more susceptible to the inhibitor, is assayed at pH 7. At this pH the $-PO_3H_2$ group of 3-phosphoglycerate is largely doubly charged, but the $-PO_3H_2$ group of the phosphonate analogue is only 26% doubly charged (pK values 6.20 and 7.45 respectively; Orr & Knowles, 1974). Both mutases are optimally active when the phospho group of the substrate is doubly charged, and with these considerations alone it might be expected that the apparent K_i for the cofactor-dependent mutase would be a factor of 4-fold greater than the apparent K_i for the independent enzyme. In fact the apparent K_i is

about 14-fold smaller, and thus the two enzymes differ over 50-fold in their sensitivity to substitution of $-\text{CH}_2-\text{PO}_3^{2-}$ for $-\text{O}-\text{PO}_3^{2-}$.

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