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Reaction of 2-azido-ATP with β subunits in the F_1-adenosine triphosphatase of *Escherichia coli*

Philip D. Bragg and Cynthia Hou

Department of Biochemistry, University of British Columbia, Vancouver (Canada)

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The three β subunits of the *Escherichia coli* F_1-ATPase react independently with chemical reagents (Stan Lotter, H. and Bragg, P.D. (1986) Arch. Biochem. Biophys. 248, 116–120). Thus, one β subunit is readily cross-linked to the ε subunit, another reacts with *N,N'*-dicyclohexylcarbodiimide (DCCD), and the third one is modified by 4-chloro-7-nitrobenzofurazan (NbfCl). The relationship of the binding site for 2-azido-ATP to the three types of β subunit recognized by chemical labeling was examined. The binding site for 2-azido-ATP was not associated with a specific type of β-subunit. There was no relationship between the site of nucleotide and the association of the ε subunit with a particular β subunit. It is concluded that the presence of the ε subunit (possibly in association with the other minor subunits) does not determine the position of the catalytic site. The possibility that the lack of a specific relationship between the 2-azido-ATP binding site and a specific β subunit was due to turnover of the enzyme, making each β a catalytic site in turn, could not be entirely rejected. However, the rate of hydrolysis of 2-azido-ATP by the DCCD-modified ATPase was very low in the presence of EDTA, and was likely due to catalysis at single sites.

Introduction

The F_1-ATPase of *E. coli* consists of five different types of subunit, designated α–ε, in a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$. Each of the subunits behaves characteristically in reactions with chemical reagents [1–4]. Thus, one of the β subunits reacts with NbfCl and IAANS but not with DCCD. Another of the β subunits reacts with DCCD but not with NbfCl or IAANS. The third β subunit does not react with the above reagents but is readily crosslinked to the ε subunit by EDC. It is not known whether the asymmetry in the reactions of the β subunits is a consequence of a permanent structural asymmetry in the F_1-ATPase [5,6] or is a measure of the transient asymmetry occurring during the catalytic cycle of the enzyme as required by the binding change mechanism of Boyer and co-workers [7].

Previous work by ourselves and others has shown that, in addition to tightly bound nucleotides, the *E. coli* F_1-ATPase has a single high-affinity binding site for ATP, as well as sites of lower affinity [8–10]. The role of this site in the catalytic mechanism of ATP hydrolysis is unclear, although it likely represents the first site at which added ATP binds and is hydrolyzed, the hydrolytic products being subsequently released on the binding of ATP at a second site [11]. In this paper we have examined the relationship of this ATP-binding site to the various types of β subunit detected by chemical labeling using 2-azido-ATP as a photoactivatable analog of ATP. Our results do not show a specific relationship of the 2-azido-ATP-binding site to one type of β subunit.

Materials and Methods

Preparation of F_1-ATPase

The F_1-ATPAse of *E. coli* ML308-225 was prepared as described previously [12], except that the fractions from the aminohexyl-Sepharose 4B column were applied to a sucrose gradient prepared in a buffer consisting of 0.1 M triethanolamine/0.5 mM EDTA/0.5 mM dithiothreitol, supplemented where indicated with 1 mM ATP, and adjusted to pH 7.5 with HCl.

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; IAANS, 2-(4-iodoacetamidoaniline)naphthalene-6-sulfonic acid; Mops, 4-morpholinepropanesulfonic acid; NbfCl, 4-chloro-7-nitrobenzofurazan.

Correspondence: P.D. Bragg, Department of Biochemistry, University of British Columbia, 2146 Health Sciences Mall, Vancouver, B.C., Canada, V6T 1W5.

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Chemical modification of F_1-ATPase

Cross-linking of β to ϵ subunits with EDC was carried out as follows. The enzyme in triethanolamine/EDTA/diethiothreitol/20% w/v sucrose buffer (pH 7.5) was incubated with 10 mM EDC at 20 °C for 60 min in the presence of 5 mM ATP and 2.5 mM $MgCl_2$. The excess reagents were removed by use of centrifuged 1 ml columns of Sephadex G-50 equilibrated with triethanolamine/EDTA buffer (pH 7.5) containing 10% (v/v) glycerol [13].

The F_1-ATPase was photolabeled with 2-azido-ATP as follows. F_1-ATPase in thiethanolamine/EDTA buffer (pH 7.5) containing 20% (w/v) sucrose or 10% (v/v) glycerol was incubated in the dark for 30–60 min with the indicated concentration of 2-azido-ATP and then irradiated for 1 min at a distance of 15 cm with a Spectroline EF 280C ultraviolet light source. Remaining reagent was removed in some experiments by use of centrifuged 1 ml columns of Sephadex G-50 equilibrated with the appropriate buffer for further manipulations.

The conditions for modification of the F_1-ATPase with NbfCl and DCCD are described in Refs. 2–4. Briefly, DCCD (0.1 mM) treatment was carried out for 1 h at 20 °C in 100 mM Mops (pH 6.55) containing 10% (v/v) glycerol and 0.5 mM EDTA. Treatment with 0.5 mM NbfCl was performed for 30 min at 20 °C in 50 mM triethanolamine/0.5 mM EDTA/10% glycerol buffer (pH 7.5). Transfer of the Nbf group from tyrosine to lysine residues was achieved by transferring the NbfCl-treated enzyme to triethanolamine/EDTA/glycerol buffer (pH 9) using a centrifuged column equilibrated with the latter buffer. The reequilibrated enzyme was incubated at 37 °C for 1 h.

Stoichiometry of binding of 2-azido-[β,γ-^{32}P]ATP by F_1 ATPase

ATPase (9.6 nmol/ml) was incubated at 20 °C for 60 min in a buffer containing 0.1 M triethanolamine/0.5 mM EDTA/0.5 mM dithiothreitol/20% sucrose (pH 7.5) with 2-azido-[β,γ-^{32}P]ATP at concentrations from 2 to 300 μM. The final volume was 50 μl. After incubation the samples were freed of unbound nucleotide by use of centrifuged 1 ml columns of Sephadex G-50 (13) equilibrated with 0.1 M triethanolamine/0.5 mM EDTA/10% glycerol buffer (pH 7.5). Samples of the effluent were assayed for radioactivity and protein.

Gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [14]. Isoelectric focusing gels were run in one dimension using the O'Farrell system [15] modified as described in Ref. 16. Proteins were stained with Coommassie blue [17]. Gels to be scanned were air-dried between two sheets of Bio-Rad dialysis membrane gel slab backing. The intensity of staining was measured with a LKB Ultrascan XL scanning densitometer. Gels containing radioisotopes were soaked for 20 min in Amplify (Amersham), dried under reduced pressure, and exposed at −70 °C to Kodak XAR-5 film.

Assays

ATPase activity was measured at 37 °C in the presence of 2.5 mM $MgCl_2$ as described previously [18]. Protein was determined by the method of Bradford [19], with bovine serum albumin as a standard.

Chemicals

The following materials were supplied by the companies indicated Sigma: DCCD, NbfCl, EDC, ATP. Pharmacia: ampholytes, Sephadex G-50. Amersham: [^{14}C]DCCD, [^{32}P]phosphoric acid, Amplify. Research Products International Corporation: [^{14}C]NbfCl.

The ATP analog, (β,γ-^{32}P)-labeled or nonradioactive 2-azido-ATP was prepared from 2-azido-AMP, kindly provided by Dr. Paul Boyer, as described by Melese and Boyer [20].

Results

Incubation of the F_1-ATPase with 2-azido-ATP at concentrations up to 250 μM in the absence of Mg^{2+} revealed a single binding site for the ATP analog (Fig. 1) (0.96 mol 2-azido-ATP bound/mol F_1; K_d, 10.3 μM).

Covalent labeling of the β subunit with 2-azido-ATP

The F_1-ATPase was incubated with 200 μM 2-azido-ATP, in the absence of Mg^{2+}, for 45–60 min before

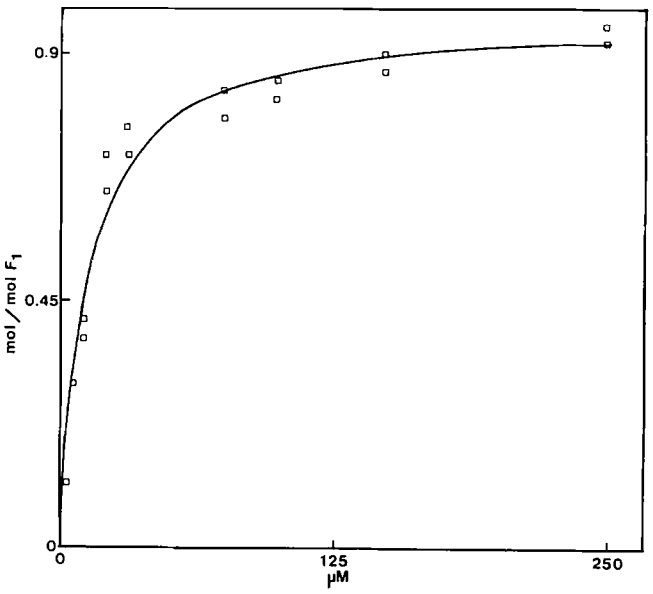

Fig. 1. Binding of 2-azido-[β,γ-^{32}P]ATP to ATPase. The method is described in Materials and Methods. The line is the computer-derived best fit to the data points for a K_d of 10.3 μM and 0.96 mol 2-azido-ATP bound/mol ATPase.

being irradiated with ultraviolet light for 1 min. There was a 10% loss of enzyme activity. The samples were exmained by isoelectric focusing after dissociation of the subunits with 1% SDS. A portion (about one-third) of the β subunits was found to migrate on the isoelectric focussing gels as having acquired two negative charges. This can be seen clearly in Fig. 2, lanes 2 in spite of the presence of different isoelectric forms of the β subunit. Modification of the other subunits was not detected.

Relationship of the 2-azido-ATP-labeled β subunit to the asymmetrically labeled β subunits

The F_1-ATPase labeled with 2-azido-ATP was subsequently treated with [^{14}C]DCCD or [^{14}C]NbfCl to study the relationship of the 2-azido-ATP-labeled subunit to those β subunits which react specifically with DCCD or NbfCl [1–4]. The enzyme was also labeled first with DCCD or NbfCl and then with 2-azido-ATP. (Treatment with DCCD and NbfCl resulted in the loss of 99 and 85% of ATPase activity, respectively.) The subunits were then examined by isoelectric focussing on polyacrylamide gels. The results of two sets of experiments are shown in Fig. 2. In lanes 5 about one-third of the β subunits was converted to a species which migrated having acquired one positive charge. This is due to the derivatization of a negatively charged glutamic acid residue by the carbodiimide [21]. Subsequent treatment of the labeled F_1-ATPase with 2-azido-ATP resulted in the intensitification of the band in the -1 position (Fig. 2, lanes 6). That this new band was due to the acquisition of two negative charges by the DCCD-labeled $+1$ species is shown by the presence of radiolabel from [^{14}C]DCCD in this band (Fig. 2, lanes 6, autoradiographs on the right-hand side). The same result was obtained if the enzyme was treated first with 2-azido-ATP (Fig. 2, lanes 3). By contrast, when the above experiment was repeated using [^{14}C]NbfCl, neither the 2-azido-ATP-modified β subunit or the NbfCl-modified subunit were further substituted by the other reagent.

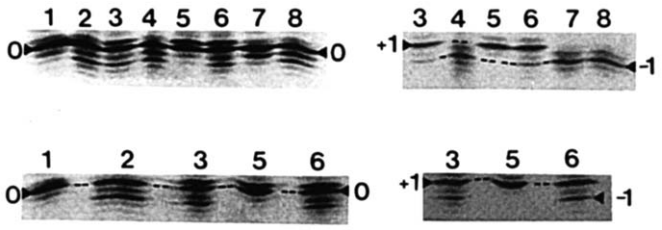

Fig. 2. Incorporation of 2-azido-ATP, [^{14}C]Nbf-Cl and DCCD into β subunits of ATPase. The ATPase was treated as described in Materials and Methods, and the labeled β subunits separated on an isoelectric focussing gel. The gel was stained with Coomassie blue (left-hand side panels) or exposed to X-ray film (right-hand side panels). The position of the major isoelectric species of the β subunits is designated 0 (zero). The number of positive or negative charges due to incorporation of labels is indicated. The lanes are 1, untreated F_1; 2, F_1 treated with 0.1 mM (upper panel) or 0.5 mM (lower panel) 2-azido-ATP; 3, F_1 treated with 2-azido-ATP and then with [^{14}C]NbfCl; 5, F_1 treated with [^{14}C]DCCD; 6, F_1 treated with [^{14}C]DCCD and then with 2-azido-ATP; 7, F_1 treated with [^{14}C]NbfCl; 8, F_1 treated with [^{14}C]NbfCl and then with 2-azido-ATP.

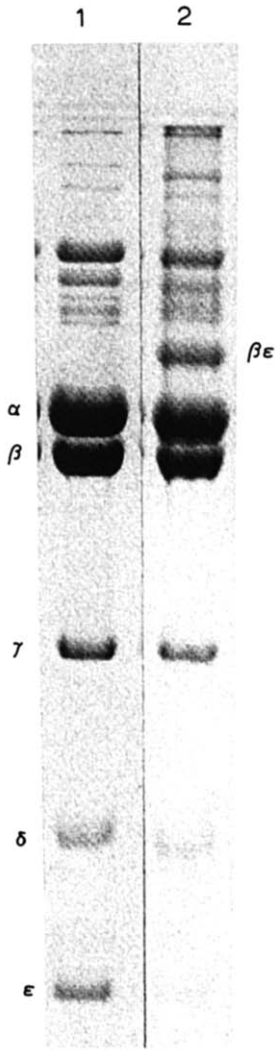

Fig. 3. Cross-linking of β and ϵ subunits of ATPase labeled with 2-azido-[β,γ-^{32}P]ATP. The ATPase was labeled with 2-azido-ATP (lane 1) before cross-linking with EDC (lane 2). The gel was stained with Coomassie blue. The positions of migration of the ATPase subunits are indicated.

The relationship of the binding site for 2-azido-ATP to the β subunit which is readily cross-linked to the ϵ subunit by treatment with EDC [1–3] was next examined. Since there was only about 70% conversion to the $\beta\epsilon$ cross-linked species (Fig. 3), the F_1-ATPase was photolabeled with [β,γ-^{32}P]2-azido-ATP, cross-linked with EDC, and the products then separated by SDS gel electrophoresis. The β and $\beta\epsilon$ bands, revealed by staining with Coomassie blue, were excised and their radioactivity was measured. The degree of conversion of β to $\beta\epsilon$ was measured by scanning the Coomassie blue-stained gel in a densitometer. The experiment was repeated modifying the F_1-ATPase with 2-azido-ATP before cross-linking with EDC.

TABLE I

Distribution of radioactivity from 2-azido-[β,γ-^{32}P]ATP in EDC-cross-linked ECF$_1$

The labeling and cross-linking were carried out as described in Materials and Methods. The distribution of radioactivity was determined in β and βε bands excised from polyacrylamide gels. The ratio of β : βε was determined by densitometry of the stained bands on polyacrylamide gels.

Order of reactions	Radio of radioactivity [a] β : βε	Ratio of protein stain β : βε
Photolabeling then cross-linking	6.21 ± 1.13	4.32 ± 1.36
Cross-linking then photolabeling	3.29 ± 1.02	

[a] Mean ± S.D. (nine separate samples).

The ratio of radioactivity in β compared with that in the βε cross-linked species was 6.2 : 1 when photolabeling preceded cross-linking and 3.3 : 1 when cross-linking preceded photolabeling (Table I). In both instances, the ratio of β : βε was about 4.3 : 1 as judged from the intensity of staining of the protein bands with Coomassie blue.

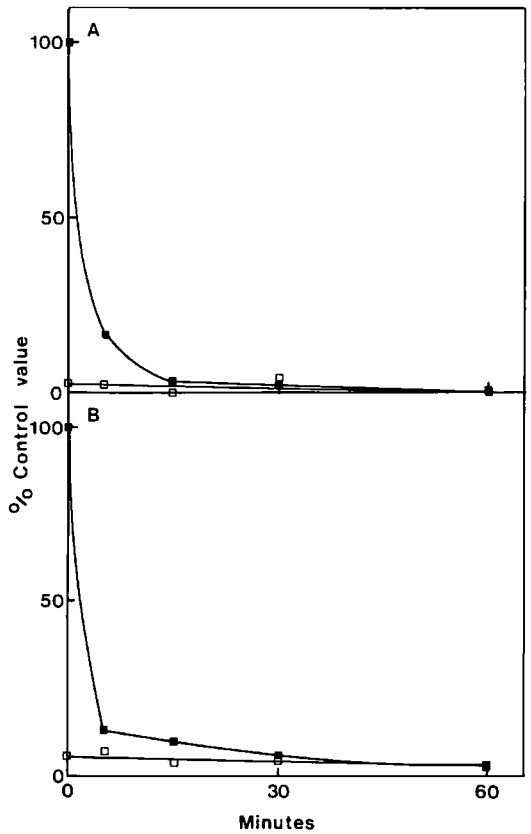

Fig. 4. Time-course for the inhibition of hydrolysis of ATP (upper panel) and 2-azido-ATP (lower panel) by 0.1 mM DCCD. The ATPase activity was assayed in the presence of 0.5 mM EDTA (open symbols) or 0.1 mM $MgCl_2$ (closed symbols). The nucleoside triphosphates were present at a concentration of 0.2 mM.

Inhibition of F$_1$ ATPAse by DCCD

The extent of hydrolysis of ATP and 2-azido-ATP by normal and DCCD-treated F_1-ATPase was examined. Unmodified enzyme in the presence of 0.1 mM Mg^{2+} and 0.2 mM nucleoside triphosphate, hydrolyzed 2-azido-ATP at about 60% of the rate of ATP hydrolysis. The effect of DCCD treatment on the catalytic activity of F_1 is shown in Fig. 4. The hydrolysis of ATP in the presence of Mg^{2+} was almost completely inhibited following 30–60 min of treatment with 0.1 mM DCCD. The hydrolysis of 2-azido-ATP in the presence of Mg^{2+} was reduced by 97% after 60 min reaction with DCCD. This was about the level of hydrolysis observed when the reaction was carried out in the presence of 0.5 mM EDTA. Clearly, little Mg^{2+}-requiring ATPase activity remains after 30–60 min treatment of the enzyme with DCCD. The significance of the low residual activity isnot clear. However, sufficient hydrolytic activity remained to cause complete hydrolysis of 2-azido-ATP during the preincubation period prior to irradiation, even with the DCCD-treated ATPase.

Discussion

The F_1-ATPase of *E. coli* in the presence of 0.5 mM EDTA showed a single high-affinity binding site for 2-azido-ATP. The observed K_d value of 10.3 μM may be compared with that of 5 μM for ATP [10] and 7 μM for 2-azido-ADP [22]. Photolysis of 2-azido-ATP resulted in labeling of the β subunits only. This agrees with the conclusions of Issartel et al. [22] and Wise et al. [23]. Under the conditions of our experiment, it is likely that the ADP derivative is linked to the enzyme due to hydrolysis of 2-azido-ATP, even in the presence of EDTA. It is interesting that labeling of the ATPase had only a limited effect on enzyme activity. This suggests that a non-catalytic site was labeled in our experiments.

An attempt to relate the binding site for 2-azido-ATP to a specific β subunit identified by chemical labeling led to the following results. The azido-nucleotide could label the β subunit modifiable by DCCD, as well as the unreacted β subunit. It did not react with the β subunit labeled by NbfCl (Fig. 2). The azido-nucleotide could also label the β subunit cross-linked to the ε subunit, but it also labeled the unreacted β subunit (Table I). The latter data may be interpreted as follows. Table II shows that expected distribution of radioactivity from 2-azido-[β,γ-^{32}P]ATP in β and βε species where only the β subunit which cross-links to the ε subunit is labeled, where only the β subunits not reacting with ε are labeled, and where the azido-nucleotide does not discriminate between any of the β subunits. Allowance is made for 50 and 100% conversion to βε. The results suggest that the binding of 2-azido-ATP does not discriminate between the various types of β subunit. This result is consistent with the double labeling of β sub-

TABLE II

Possible ratios for distribution of radioactivity from 2-azido-[β,γ-^{32}P]ATP in β and βε

β * is the specific β subunit which cross-links to ε. 1 mol azido-ATP/mol ECF_1.

Condition	% Cross-linking of βε	Ratio β : βε	
		radio-activity	protein
Only β * labeled	100	0:1	2:1
	50	0.5:0.5	2.5:0.5
Only β labeled	100	1:0	2:1
	50	1:0	2.5:0.5
No discrimination between β and β *	100	0.66:0.33	2:1
	50	0.83:0.17	2.5:0.5
Found (2-azido-ATP, then EDC)		6.2:1	4.3:1
Found (EDC, then 2-azido-ATP)		3.3:1	

unitsby DCCD and the azido-nucleotide. The inability of 2-azido-ATP to react with the NbfCl-modifiable β subunit may be due to competition between the reagents for a binding site. It is of interest that the model of Duncan et al. [24] for the tertiary folding topology of the adenine nucleotide-binding site of the β subunit places the 2-azido-ATP binding site (tyrosine-331) close to lysine-155, to which Nbf is tranferred from tyrosine-297 after the pH 9 treatment. By contrast, the binding site for DCCD is not close to the nucleotide binding site.

A potential complicating factor in our experiments could be the incomplete labeling of the ATPase by the chemical reagents. This would generate two populations of enzyme – modified and unmodified. Treatment with DCCD resulted in almost complete loss of enzyme activity concomitant with the modification of about one-third of the β subunits. It is unlikely that much unmodified enzyme was present. By contrast, modification with NbfCl gave 85% loss of ATPase activity. Some unmodified enzyme would be present. However, this would not affect the interpretation of the results discussed above.

The lack of discrimination by 2-azido-ATP between apparently asymmetrically reacting β subunits may have two explanations. The first possibility is that the asymmetry does not affect the nucleotide-binding sites. This explanation is of interest with regards to the hypothesis that during ATP synthesis the ε or/and other minor subunits change their relationship to the β subunits during the catalytic cycle, causing each β subunit to become the active site in turn [25]. No relationship between the ε subunit and the nucleoltide-binding site was found in our experiments. It should be noted, however, that these experiments used the F_1-ATPase. The behavior of the F_1F_0-ATPase has yet to be determined. The second possibility is that each β subunit becomes the azido-nucleotide binding site sequentially during the catalytic cycle. This could occur if the enzyme was turning over during the photolabeling. We attempted to assess the likelihood of this possibility under conditions where the enzyme was modified by DCCD, since DCCD is believed to block the cooperativity of F_1-ATPase [26]. In the presence of EDTA, the conditions of the photolabeling, the DCCD-treated enzyme showed nearly zero activity with ATP. About 3% of the activity seen in the presence of $MgCl_2$ was observed with 2-azido-ATP as substrate. Although the residual avtivity was very low, it was sufficiently significant for complete hydrolysis of the added 2-azido-ATP to 2-azido-ADP to have occurred during the preincubation period prior to photolysis. If this hydrolysis were due to catalysis at a single site, as seems likely, then there would not have been sequential cycling of the catalytic sites between the β subunits. This leads to the conclusion that the binding site for 2-azido-ATP is not associated with a specific β subunit recognized by chemical probes.

It is interesting to compare these results with those of Melese and Boyer using the chloroplast F_1-ATPase [20]. These workers found that 2-azido-ATP labeled different β-subunits when the azido-nucleotide was permitted to react before DCCD. By contrast, DCCD modification before 2-azido-ATP labeling led to labeling of the same β subunit by both reagents. The authors attributed the double-labeling to residual turnover of the DCCD-modified chloroplast F_1. In our experiments, we could see no marked difference dependent on the order of labeling by the two reagents. Possible significant differences between our experiments and those of Melese and Boyer are that the chloroplast F_1 was incubated with 2-azido-ATP in the presence of Mg^{2+}, and that DCCD modification was performed in the presence of ATP. In our experiments, ATP and Mg^{2+} were excluded and 0.5 mM EDTA was present, to minimize the possible catalytic turnover of the enzyme.

Acknowledgements

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