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Brief Communication

In Vivo Resolution of Conflicting In Vitro Results: Synthesis of Biotin from Dethiobiotin Does Not Require Pyridoxal Phosphate

Ahmed M. Abdel-Hamid[1] and John E. Cronan[1,2,*]
[1]Department of Microbiology
[2]Department of Biochemistry
University of Illinois, Urbana, IL 61801, USA
*Correspondence: j-cronan@life.uiuc.edu
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SUMMARY

The source of the biotin sulfur atom remains a contested point in studies of biotin synthase (BioB) in vitro. Recent reports that BioB has an intrinsic pyridoxal phosphate (PLP)-dependent cysteine desulfurase activity were tested by depleting *Escherichia coli* cells of PLP. The BioB-dependent conversion of dethiobiotin to biotin proceeded in these cells irrespective of the presence or absence of PLP.

INTRODUCTION

The ability to study enzymes in vitro is a double-edged sword. The obvious benefit is that many parameters can be manipulated to gain insight into the chemical mechanism, physiological role, and regulation of the enzyme activity. However, manipulation can also be a disadvantage in that enzymes can be forced to work in a nonphysiological manner. The advantage of studying enzymes of genetically amenable organisms is that hypotheses based on in vitro studies of an enzyme can often be tested in vivo. We previously addressed the question of the catalytic ability of the BioB protein (biotin synthase) responsible for the last step of biotin synthesis (Figure 1) in this way [1]. Despite consistent reports that BioB was not catalytic in vitro, we found it is catalytic in vivo, although BioB is an extremely modest catalyst (20–60 turnovers/monomer) and catalysis engenders proteolysis of the enzyme [1]. We report modifications of the experimental approach used in that work to ask if pyridoxal phosphate (PLP) is required for insertion of the biotin sulfur atom.

E. coli BioB has long been a bête noire of mechanistic enzymology. Extensive attempts to obtain sulfur insertion in vitro failed until Ifuku and coworkers [2] demonstrated biotin synthesis from DTB in a cell-free extract that required S-adenosylmethionine (AdoMet). This led to a defined system containing NADPH, flavodoxin and flavodoxin reductase as the electron transfer system, DTB, AdoMet, and BioB plus a reducing environment [3]. The discovery that SAM was absolutely required for biotin synthesis and was not the sulfur donor [3] strongly suggested that BioB was a member of the (then) small family of ''radical SAM'' enzymes, which has proven to be the case [4]. The lack of BioB catalysis in vitro [4–6] means that reactions must contain large quantities of BioB to allow detection of the biotin produced. Thus, the requirement for purity of BioB is more stringent than for a normally robust enzyme where contaminants introduced with the purified enzyme preparation will generally be of very low concentration and irrelevant. However, obtaining highly purified BioB is problematical because the enzyme is intrinsically unstable, and thus most purification protocols are a compromise between chemical purity and activity of the protein. Thus, it is not surprising that many conflicting reports are found in the BioB literature. A prime example is the source of the biotin sulfur atom. The currently accepted view is that the sulfur atom is derived from the [2Fe-2S] cluster of BioB (the enzyme also contains a [4Fe-4S] cluster involved in generation of the deoxyadenosyl radical) [4–6] based on experiments in which isotopically labeled biotin was produced in vitro when the [Fe-S] centers of BioB were labeled with ^{35}S or ^{34}S either in vivo [7] or in vitro [8]. Recently, BioB reconstituted with Se in place of S gave selenobiotin [9]. However, in vitro incorporation of cysteine-derived sulfur into biotin by BioB has also been reported, although there is marked disparity among these reports. In some cases, the levels of incorporation from cysteine appear much lower than stoichiometric [10, 11], whereas others report that transfer of label from [^{35}S]cysteine to DTB failed to occur [3, 8]. Finally, stimulation of biotin synthesis by addition of either cysteine stereoisomer is reported [12]. A possible explanation for some of these data arose from the recent reports that BioB has an intrinsic pyridoxal 5′-phosphate (PLP) dependent cysteine desulfurase activity [13, 14]. This activity was proposed to mobilize sulfide from cysteine with the sulfide becoming the biotin sulfur atom via a protein-bound persulfide [13, 14]. However, a subsequent BioB crystal structure showed neither PLP nor an obvious PLP binding site [15] and others report no PLP stimulation of the BioB reaction [16, 17]. A caveat is that crystallization necessarily selects for a single protein species and there is evidence that more than one form of BioB might exist. For example, it has been reported that biotin synthesis occurs concomitantly with collapse of the BioB [2Fe-2S] cluster [18], whereas another laboratory reports

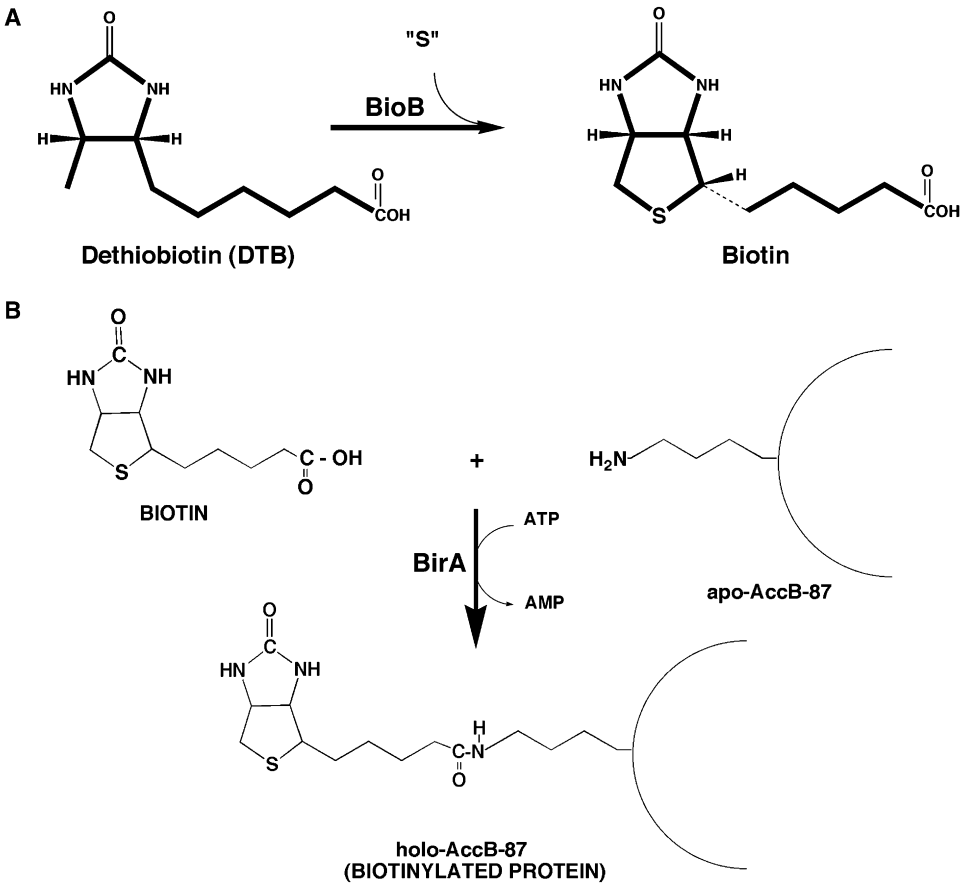

Figure 1. The Biotin Synthase and Biotin Protein Ligase Reactions
(A and B) The biotin synthase (BioB) reaction (A) and the ligation-mediated assay for biotin synthesis (B). DTB is not a substrate for the BirA ligase reaction in vivo [1] and is a much poorer substrate (ca. 50,000-fold) than biotin in vitro [21].

that biotin synthesis lags behind collapse of the [2Fe-2S] cluster suggesting the possibility of another intermediate such as a persulfide [19]. We have addressed the question of the reported BioB cysteine desulfurase activity and its possible role in the origin of the biotin sulfur atom and report that *E. coli* cells unable to synthesize PLP remain fully proficient in conversion of DTB to biotin upon depletion of the intracellular PLP pool to undetectable levels.

RESULTS AND DISCUSSION

The experimental design was similar to that previously used [1] except that the *E. coli* host strain was blocked in the synthesis of pyridoxal (due to a null deletion/insertion mutation of *pdxH* which encodes pyridoxine 5′-phosphate oxidase [20]) as well as biotin (due to deletion of the *bioABFCD* operon). The *pdxH* mutation was chosen to block PLP synthesis because PdxH is required for both the PLP biosynthetic and salvage pathways [20]. Biotin synthesis was measured by the covalent attachment of biotin to the 87-residue biotinoyl domain of *E. coli* AccB (AccB-87) catalyzed by the *E. coli* BirA biotin protein ligase [1]. The assay depends on the fact that DTB is not a BirA substrate in vivo [1] and a much poorer (ca. 50,000-fold) substrate for the BirA ligase than biotin in vitro [21]. The BioB source was an N-terminally hexahistidine-tagged protein produced from an arabinose-inducible *araBAD* promoter on a multicopy plasmid [1]. The N-terminally hexahistidine-tagged AccB87 biotinoyl domain and BirA ligase were expressed from the powerful IPTG-inducible T7 promoter on a compatible multicopy plasmid [1]. Cultures of the Δ*bioABFCD* Δ*pdxH* strain transformed with the two plasmids were grown overnight on a vitamin-free medium plus biotin and pyridoxal. The cells were collected, washed, and resuspended in medium free of both biotin and pyridoxal and starved. Expression of the hexahistidine-tagged AccB-87, BirA, and BioB proteins was then induced. Protein synthesis was blocked, and the cultures were split into fourths that received DTB, DTB plus pyridoxal, biotin, or water. After further incubation, biotin synthesis was detected by western blotting with a streptavidin-enzyme conjugate or by separation of the holo- and the apo domain on native PAGE and western blotting against anti-pentahistidine antibody. Biotinylation was also assayed by measuring incorporation of radioactivity into protein (holo Acc87 plus AccB) in cultures

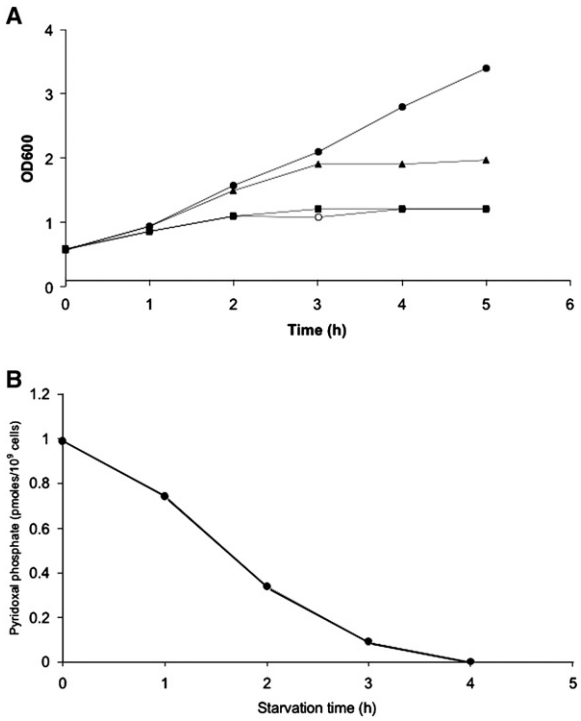

Figure 2. Pyridoxal Starvation

(A) Shows the growth of strain supplemented with both pyridoxal and biotin (solid circles) supplemented with only biotin (pyridoxal starvation) (solid squares), supplemented with only pyridoxal (biotin starvation) (solid triangles), and with neither supplement (open circles).

(B) Depletion of the PLP pool upon pyridoxal starvation. Three separate experiments (two by colorimetry and one by HPLC assay) gave almost identical extents and rates of depletion.

supplemented with radioactively labeled DTB. We used a medium rich in amino acids (but devoid of vitamins) to mitigate effects of pyridoxal starvation on protein synthesis (although protein turnover would provide low levels of amino acids in the absence of PLP). Upon starvation of cultures for pyridoxal the growth of the cultures gradually slowed and then ceased after about 3 hr of starvation (Figure 2). The intracellular PLP levels of the starved cells released by acid treatment were then assayed by activation of the apo form of *E. coli* tryptophanase by PLP. After 4 hr of pyridoxal starvation, the PLP levels had declined >100-fold to levels below the sensitivity of the assays (<0.004 pmol/10^9 cells) (Figure 2). The rapid decrease in cellular PLP levels might seem surprising since the starved cultures showed only minimal growth during the starvation period and thus the decrease could not be due to dilution by growth. However, in a seemingly perverse process, *E. coli* excretes PLP to the growth medium such that most of the PLP of a growing culture is found in the medium [22] from which it cannot be recovered [23]. It should also be noted that starvation for pyridoxal and/or biotin is not bactericidal. Upon restoration of the supplements, growth resumed within 30 min, and the intracellular PLP pool returned to about one-third of the prestarvation level within 1 hr. The restoration of the PLP pool was expected because only an ATP-dependent phosphorylation by either of two pyridoxal kinases is required to convert pyridoxal to PLP. The starved cells must be rich in ATP because upon biotin addition the BirA ligase quantitatively converted apo-AccB-87 to the biotinylated form (see below). Despite the fact the cell cultures were metabolically compromised by pyridoxal starvation, the hexahistidine-tagged proteins of interest were produced at readily accessible levels and their production was tightly regulated (Figure 3B). This allowed us to test whether or not the conversion of DTB to biotin required PLP (Figure 3A). In the cultures in which all three proteins were expressed, addition of DTB resulted in the production of a streptavidin-reactive protein regardless of the presence or absence of pyridoxal. Production required BioB expression as well as that of AccB-87 and BirA (Figure 3A). Addition of biotin showed that the levels of acceptor protein and ligase were present in great functional excess (Figure 3A). A second assay used the increased negative charge of the acceptor domain upon biotinylation of the lysine residue and showed the expected [1] shifted band upon addition of DTB irrespective of pyridoxal addition (Figure 3C). These data indicated that conversion of DTB to biotin proceeded in the absence of PLP and was not increased by restoration of PLP. This was confirmed by a third assay, labeling starved cells with [^{14}C]DTB in the presence or absence of pyridoxal and assaying protein-bound radioactivity (via conversion of DTB to biotin which can be attached to AccB-87) (Figure 4). These data showed that essentially the same levels of conversion of DTB to biotin occurred in the presence or absence of PLP consistent with the data of Figure 3. Note that because BioB is degraded upon catalysis [1], kinetic analysis is precluded.

We conclude, in agreement with in vitro results of others [16, 17], that the BioB reaction does not require PLP in vivo. Hence, in vitro results to the contrary [13, 14] can probably be attributed to unrecognized components of the complex reaction mixtures required for in vitro assay of BioB activity. Our work also raises the possibility that PLP may not be strictly required for production of sulfide from cysteine in *E. coli*. This is because BioB contains (and requires) two [FeS] clusters for activity [4, 6, 15]. The current picture of [FeS] cluster assembly in *E. coli* is that the sulfur atoms are derived by PLP-dependent cysteine desulfurases via a protein bound persulfide [24]. The Isc system with its cysteine desulfurase, IscS, is thought to be the major pathway for de novo cluster biosynthesis, whereas the Suf system with its cysteine desulfurase, SufS, is thought to function in repair of clusters damaged by oxidation or iron starvation. Mutant strains lacking IscS show deficiencies in some [FeS] cluster-requiring enzymes, whereas other such enzymes retain much of their activity [25, 26]. One possible explanation is that the *suf* operon becomes derepressed under Isc-deficient conditions and SufS functionally replaces IscS. Indeed, there is a regulatory connection between the two pathways because the [2Fe-2S] cluster transcription factor IscR acts as a repressor of the *isc* operon when its cluster is intact but as an activator of the *suf* operon

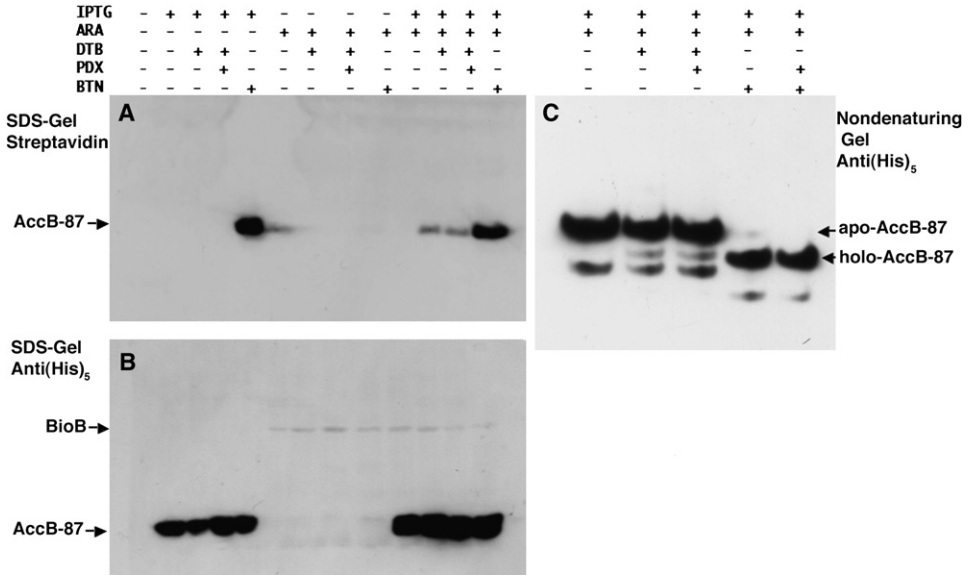

Figure 3. Synthesis of Biotin from DTB

In (A) and (B), two identical SDS-gels were run in parallel, and the proteins were transferred to Immobilon P membranes. The proteins of the membrane in (A) were visualized by western blotting with a streptavidin conjugate, whereas the proteins of the other membrane (B) were visualized with an anti-pentahistidine antibody. In (A), the band in the sixth lane from the left is due to leakage from the adjacent lane. In (C), a nondenaturing gel was run on a different set of reactions. The proteins of the gel were transferred to an Immobilon P membrane and were visualized by western blotting with the anti-pentahistidine antibody. All of the protein samples of the figure had been purified by elution from a Ni-cheleate column prior to electrophoresis. The fastest moving band in the lanes of (C) may be an AccB-87 deamidation product. Electrospray mass spectroscopy gave masses of 11,656.0 (calc. 11,655.3) and 11,882.0 (calc. 11,881.3) for the apo and biotinylated AccB-87 forms, respectively. Very similar results were obtained in three additional experiments. Moreover, a series of experiments done with a set of plasmids in which the promoters were reversed relative to the configuration used above also showed very similar levels of biotin synthesis from DTB (data not shown) in the presence and absence of PLP. However, in these experiments, the biotin ligation assay may have been somewhat limited by the lower levels of AccB-87 expression.

when the cluster has been degraded [27]. However, this cannot explain the residual [Fe-S] enzyme activities of *ΔiscS* strains because strains carrying a deletion of the entire *isc* operon (including *iscR*) have the same growth phenotype as *ΔiscS* strains [26]. These uncertainties together with our data raises the possibility that *E. coli* may contain undiscovered cysteine desulfurases or that there is a sufficient supply of sulfide present in vivo to allow cluster assembly from free cytosolic sulfide and iron (which proceeds fairly readily in vitro). One possibility for undiscovered cysteine desulfurases is that formation of a Schiff base with cysteine, the role of PLP in the known cysteine desulfurases, could be performed by a posttranslationally generated N-pyruvoyl protein moiety. Indeed, this hypothesis is consistent with the recent report that in yeast mitochondria, two proteins are required for biotin synthase activity but not for de novo synthesis of its two [FeS] clusters [28].

In regards to BioB, the question of how the BioB [2Fe-2S] center is built and restored remains open. Although BioB has been reported to accept an [4Fe-4S] center from two *E. coli* Fe-S center scaffold proteins, SufA and IscA, no [2Fe-2S] center was formed [29]. It should be noted that the BioB [2Fe-2S] cluster has a novel ligand, an arginine residue [15]. However, substitution of Cys, Ala, His, or Met for the arginine residue failed to inactivate BioB [30]. The plasticity of the cluster suggests that the usual sulfur insertion pathways (the Isc and Suf systems) may not apply, and this seems to be the case. Inclusion of IscS does not allow BioB to become catalytic in vitro

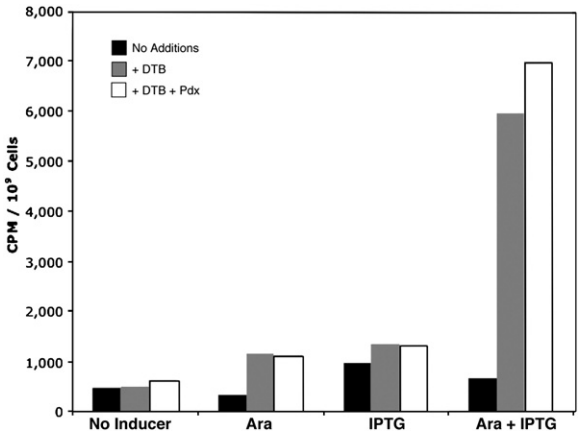

Figure 4. Conversion of [^{14}C]DTB to Biotin

[^{14}C]DTB labeled in the ureido carbon was synthesized, and its attachment to protein measured as described in Experimental Procedures. The data are given in relative terms (cpm rather than dpm) due to uncertainty in the specific activity of the DTB due to the inherent inaccuracy of the bioassay plus the possibility of exchange with atmospheric CO_2 of the $NaH^{14}CO_3$ used in DTB synthesis. The experimental error of the reported values is estimated to be ± 10%. The abscissa gives the inducer added.

[31], and *E. coli* strains having null mutations of either the *suf* or *isc* operons do not require biotin for growth (J. Imlay, personal communication). Unfortunately, *suf isc* double mutant strains are inviable so the possibility that biotin is synthesized by redundant functions of the two systems cannot be tested.

SIGNIFICANCE

The biotin synthase (BioB) reaction does not require PLP in vivo. Therefore, the biotin sulfur atom cannot be derived via an intrinsic PLP-dependent BioB cysteine desulfurase activity.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids

The Δ*pdxH::CAT* allele of strain TX2767 [20] was transduced into strain ER47 with phage P1*vir* [1] to give strain AH14 into which plasmids pER30 and pER35 [1] were introduced.

Growth Conditions

Cultures were grown overnight at 37°C in M9 basal medium supplemented with 0.2% glucose, 1% Vitamin Assay Casamino Acids (Difco), 1 mg/l thiamine, 50 mg/l ampicillin, 50 mg/l kanamycin, 30 mg/l chloramphenicol, 1 μM pyridoxal, and 4 nM biotin. The cells were collected and washed with biotin-free medium. The cells were then resuspended in the same medium as above except lacking pyridoxal and biotin and adjusted to 0.6 at 600 nm and grown to starve for both biotin and pyridoxal. After starvation for 4 hr at 37°C, cells were resuspended in the medium but containing 0.2% glycerol (in place of glucose) and plus 0.1% arabinose and 1 mM IPTG. Growth was continued for 4 hr after which protein synthesis was blocked with tetracycline HCl (60 mg/l). After 2 hr, cultures were then split into four subcultures. One subculture received water, another received 5 μM DTB, a third received 5 μM DTB plus 5 μM pyridoxal phosphate, and a fourth received 5 μM biotin. The cultures were then incubated overnight at 37°C. The cells were collected by centrifugation resuspended in lysis buffer (50 mM sodium phosphate [pH 8.0], 300 mM NaCl, 0.1 mM DTT, and 2 mM phenylmethylsulfonyl fluoride) and lysed by sonication. Protein analysis of the extracts was done as described previously [1]. The apo and holo AccB87 proteins were analyzed by ESI mass spectroscopy after dialysis against 2 mM ammonium acetate.

Protein Biotinylation

^{14}C-DTB labeled in the *ureido* moiety was synthesized from NaH$^{14}CO_3$ by using DTB synthetase [32] and assayed as described previously [33]. Cultures to be assayed for biotin synthesis were supplemented with 1 μM ^{14}C-DTB in the presence or absence of pyridoxal phosphate (1 μM) and biotinylation of AccB-87 was assayed as described previously [34].

Pyridoxal Phosphate Assays

Cells from 500 ml cultures were centrifuged and resuspended in 20 ml of 0.055 M HCl and autoclaved for 5 hr to liberate cell- and protein-bound pyridoxal phosphate [35]. Pyridoxal phosphate was assayed by the PLP-dependent activation of apo-tryptophanase followed by cleavage of tryptophan to indole measured both by a colorimetric reaction [36] and by reverse phase HPLC.

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