A new tool for studying protein structure and function

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A technique that allows for the site-specific incorporation of a large variety of unnatural amino acids into proteins has been developed. This methodology has been used to substitute amino acids with novel electronic, steric and spectroscopic properties into proteins, providing new insights into the mechanisms of protein stability, enzymatic catalysis and signal transduction.

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Introduction

Proteins are at the crossroads of virtually every biological process, including signal transduction, catalysis, gene regulation and the immune response. Yet we know little about the mechanisms whereby these biopolymers, composed of 20 simple building blocks, carry out their remarkable functions. One important technique used to probe the forces that govern protein structure and folding, biomolecular recognition and catalysis is site-directed mutagenesis [1]. This method makes it possible to substitute a specific amino acid in a protein with any of the other 19 common amino acids [2]. However, in contrast to studies of small molecules, for which analogs with virtually any structural change can be synthesized, changes in protein structure are limited to the 20 amino acids. Ideally, we would like to be able to tailor the steric or electronic properties of an amino acid in a protein to address a specific structure–function question. Mutations might include modifying the acidity, nucleophilicity or hydrogen-bonding or hydrophobic properties of an amino acid side chain; altering or restricting the protein backbone conformation; or introducing biophysical probes such as spin labels or isotopic labels site-specifically into proteins. The ability to substitute such unnatural amino acids, beyond those specified by the genetic code, would greatly expand our ability to manipulate and study protein structure and function and may allow the generation of new proteins with novel properties.

A number of methods can be used to incorporate unnatural amino acids into proteins. Solid-phase peptide synthesis, particularly recent advances in the segment synthesis/condensation approach, has allowed the synthesis of small proteins (up to 12 kDa) containing novel amino acids in milligram quantities [3–7]. Protein semi-synthesis, in which a synthetic peptide is ligated to a protein fragment to produce a full-length protein, has been used to incorporate unnatural amino acids into proteins [8,9]. This approach is complicated by the need to cleave the protein specifically at the peptide ligation site and by difficulties in coupling the protein and peptide termini selectively. Chemical modification has also been used to introduce a variety of unnatural side chains into proteins including cofactors, spin labels and oligonucleotides [10–15]. With this method, however, substitutions are largely restricted to simple derivatives of reactive amino acid side chains (e.g. lysine, cysteine, tyrosine) on the protein surface. Alternatively, biosynthetic methods that use chemically modified aminoacyl–tRNAs have been used to incorporate a number of biophysical probes into proteins synthesized *in vitro* [16,17]. This approach is limited, though, because the mutations are restricted to derivatives of the natural amino acids; the modified amino acid is substituted at multiple sites; and both the modified and natural amino acids are incorporated at the site of interest because of difficulties in removing endogenous aminoacyl–tRNA from the *in vitro* extract.

Recently, a biosynthetic approach has been developed that, for the first time, allows the site-specific incorporation of a large variety of unnatural amino acids into proteins [18,19••,20]. Briefly, the codon for the amino acid of interest is replaced with the stop codon UAG using standard oligonucleotide-directed mutagenesis [21]. A suppressor tRNA that recognizes this codon is chemically acylated with the desired unnatural amino acid. Addition of the mutagenized gene or mRNA and the aminoacylated suppressor tRNA to an *Escherichia coli* or rabbit reticulocyte extract capable of supporting protein biosynthesis generates a mutant protein containing the unnatural amino acid at the desired position (Fig. 1) [18,19••]. The details of the methodology are discussed in [22••]. In this article, we review applications in which the substitution of unnatural amino acids with novel electronic, steric and biophysical properties into proteins has provided new insights into the mechanisms of protein stability, enzymatic catalysis and signal transduction.

Abbreviations

SNase—staphylococcal nuclease; **T4L**—T4 lysozyme.

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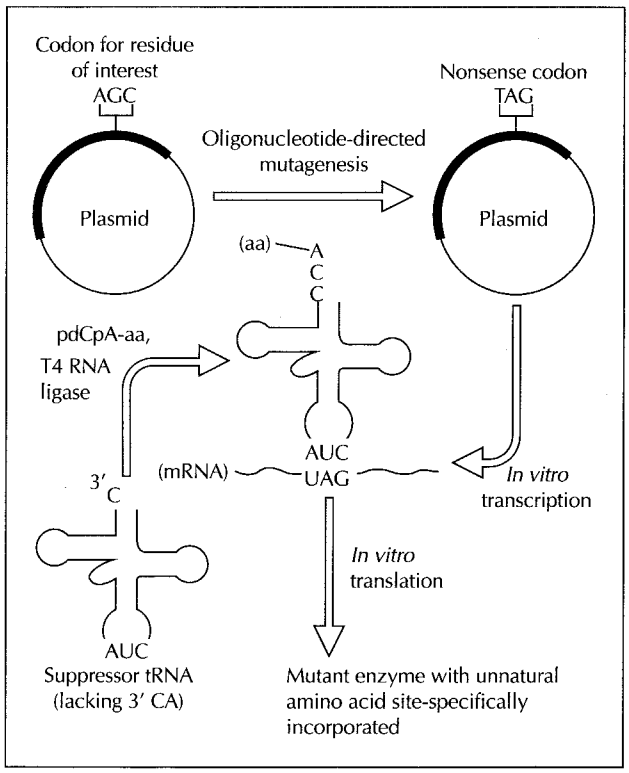


Fig. 1. A method for the site-specific incorporation of unnatural amino acids.

Biophysical probes

Several amino acids that can serve as biophysical probes have been incorporated into proteins using unnatural amino acid mutagenesis (Fig. 2). A novel spin-labeled amino acid **1**, an unnatural fluorescent amino acid **2** and an efficient cross-linking agent **3** were all introduced site-specifically at several external and internal positions in T4 lysozyme (T4L) with reasonable efficiency [23•]. Both the electron spin resonance spectrum of purified T4L containing the spin-label **1** and the fluorescence emission spectrum of purified T4L in which one of the tryptophans was replaced by 7-azatryptophan **2** were measured. With considerably more effort, a sufficient quantity of purified T4L specifically labeled at Ala82 with [^{13}C]Ala was produced to observe the proton resonances of the labeled residue selectively in both the native and denatured states using ^{13}C-filtered NMR [24•]. Finally, enough staphylococcal nuclease (SNase) containing the unnatural amino acid homoglutamate **4** at position Glu43 was obtained to determine the mutant protein's structure to 2.4 Å resolution using X-ray crystallography [25•]. The ability to incorporate probes capable of detecting local structure and dynamics into proteins site-specifically should allow more precise studies of protein folding and stability, conformational changes in proteins and the interaction of proteins with other proteins, small molecules and membranes.

'Caged' proteins

Low molecular weight caged substances, inactive precursor molecules that can be activated rapidly by photolysis, are useful in biochemical studies [26–28]. Unnatural amino acid mutagenesis provides a unique approach to the construction of photo-caged proteins. For example, the active-site residue Asp20 in T4L was substituted with the unnatural amino acid β-*o*-nitrobenzylaspartate **5** [29]. Since Asp20 is essential for catalytic activity, the resulting protein was inactive. Removal of the protecting group by photolysis of the intact, modified protein restored full catalytic activity.

In a second example, a serine residue thought to be involved in the self-spicing reaction of the protein vent DNA polymerase (Fig. 3; CJ Noren, personal communication) was replaced by *o*-nitrobenzylserine **6**. The resulting caged polymerase did not undergo the self-splicing reaction, allowing the full-length protein to be isolated. Photolysis of the unspliced protein resulted in loss of the nitrobenzyl protecting group and subsequent protein splicing. This result demonstrates unequivocally that protein splicing occurs at the post-translational level. The ability to construct caged proteins of this sort should allow a broad range of time resolved experiments relevant to catalytic mechanism, biomolecular recognition and protein folding.

Protein stability

Unnatural amino acid mutagenesis has been used to make well defined changes in proteins in the hope of gaining a more precise picture of the forces that govern protein stability [30,31]. In an examination of the importance of packing interactions in the core of a protein [32–36], replacements were made for Leu133, which lies along the edge of the largest cavity in the interior of T4L [37]. Previous attempts to stabilize T4L by increasing the packing density of the hydrophobic core via a Leu133→Phe or Ala129→Val mutation had been unsuccessful, as both mutations disrupt neighboring residues [37]. Substitution by the unnatural amino acids *S,S*-2-amino-4-methylhexanoic acid **7** and *S*-2-amino-3-cyclopentylpropanoic acid **8**, however, which were designed to fill the cavity with minimal strain, increased the thermal stability of T4L by 0.6 kcal mol^{-1} (1.9 °C) and 1.24 kcal mol^{-1} (4.3 °C), respectively [38•]. This result nicely demonstrates that amino acids that increase the bulk of buried hydrophobic surface area without any concomitant introduction of strain can increase protein stability significantly. As there is little difference in surface area between these two unnatural amino acids (Δ surface area = 0.9 Å^2), the difference in stability between the two mutants likely reflects the fact that the cyclic amino acid **8** loses less conformational entropy upon folding than does the acyclic amino acid **7**. This difference emphasizes the importance of side chain con-

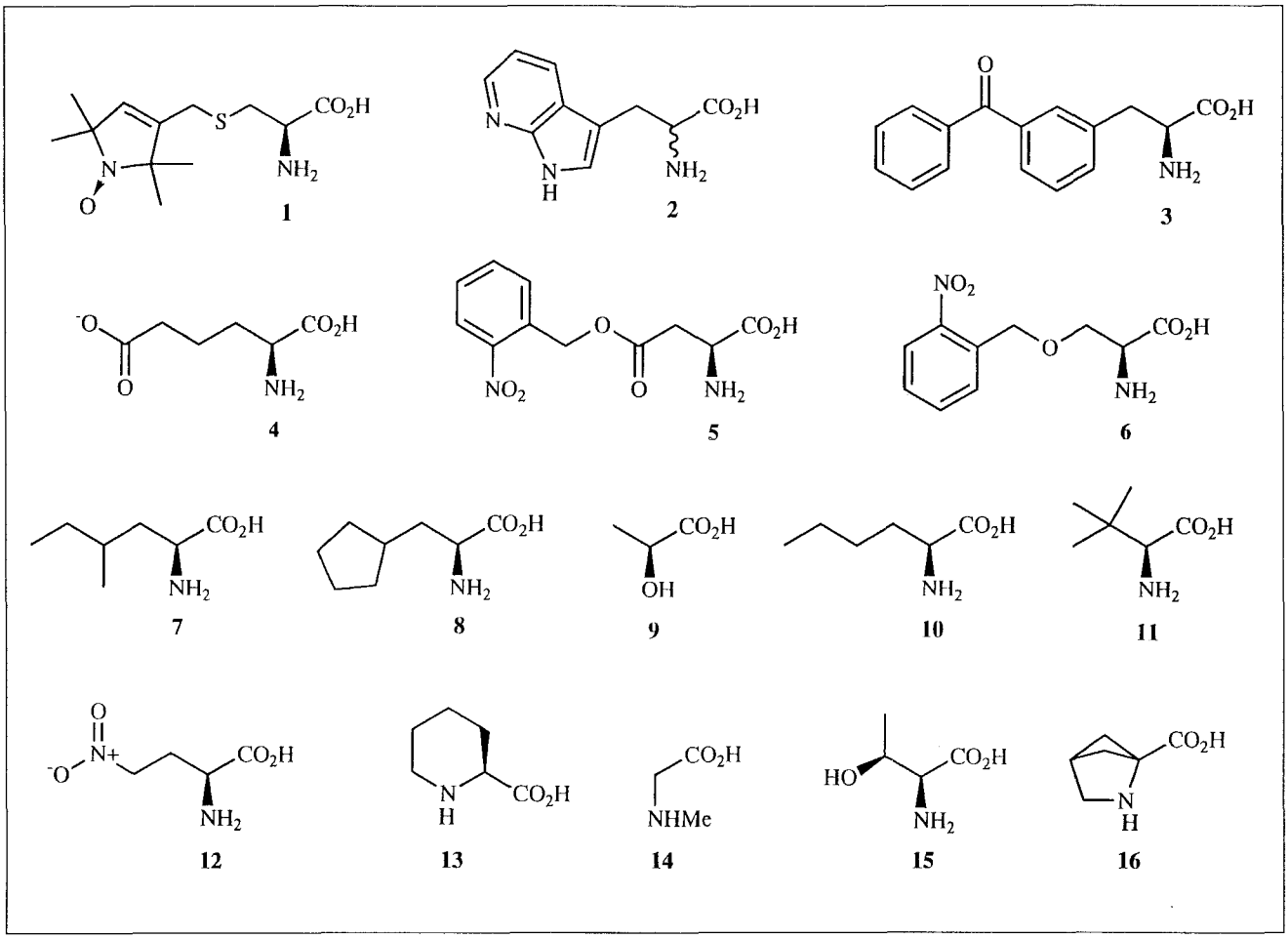


Fig. 2. Examples of unnatural aminio acids successfully incorporated into proteins. **1** *L*-2-amino-3-thiomethyl-1-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolin-3-yl)-propanoic acid; **2** 7-azatryptophan; **3** *p*-benzoylphenylalanine; **4** homoglutamate; **5** β-*o*-nitrobenzylaspartate; **6** *o*-nitrobenzylserine; **7** *S,S*-2-amino-4-methylhexanoic acid; **8** *S*-2-amino-3-cyclopentylpropanoic acid; **9** lactic acid; **10** *n*-butylglycine; **11** *t*-butylglycine; **12** *S*-4-nitro-2-aminobutyric acid; **13** pipecolic acid; **14** *N*-methylglycine; **15** threonine; **16** 2,4-methanoproline.

formational entropy, in addition to packing interactions, in determining protein stability.

A series of mutations has also been made in T4L to examine the contribution made by the polypeptide backbone to protein stability [39•]. Although the strengths of backbone hydrogen bonds have been difficult to determine, they are not generally thought to contribute significantly to the stability of the folded state of a protein [30]. In order to test this notion the surface amino acid Ala82 in T4L was replaced with its isostere lactic acid **9**. Ala82 is at a break between two helices, with the NH group and side chain exposed to water. The carbonyl of the preceding residue, Asn81, is hydrogen-bonded to the amide NH group of Lys85. The Ala82→lactate mutation effectively substitutes a good hydrogen-bond acceptor, the amide carbonyl group, with a considerably weaker hydrogen bond acceptor, the ester carbonyl group [40]. Because both esters and amides occur mainly in the *trans* conformation [41], the 1.0 kcal mol^{-1} (3.7 °C) destabilization caused by this substitution indicates that backbone amide hydrogen-bonding interactions do in fact help to stabilize proteins.

The effect of incorporating β-branched amino acids in protein α-helices has been examined by comparing the stability of mutant proteins containing alanine, *n*-butylglycine **10** or *t*-butylglycine **11** at two surface-exposed sites in the middle of two α-helices in T4L (VW Cornish, PG Schultz, unpublished data). The natural β-branched amino acids valine, isoleucine and threonine are thought to destabilize α-helices. These amino acids occur infrequently relative to amino acids such as leucine in α-helices in known protein structures, and they destabilize α-helical peptides and protein α-helices [42–48]. For example, when a series of linear and branched amino acids were incorporated into an α-helical peptide, the β-branched amino acids proved to be destabilizing relative to the linear and γ-branched amino acids [49]. In particular, substitution of *t*-butylglycine for *n*-butylglycine or alanine destabilizes the peptide by about 0.9 kcal mol^{-1}. The same substitution, however, either destabilizes T4L by 0.69 kcal mol^{-1} (2.5 °C) at site Ser44 or stabilizes the protein by 0.27 kcal mol^{-1} (1.0 °C) at site Asn68. This difference illustrates the difficulty of establishing simple rules about which factors stabilize or destabilize proteins

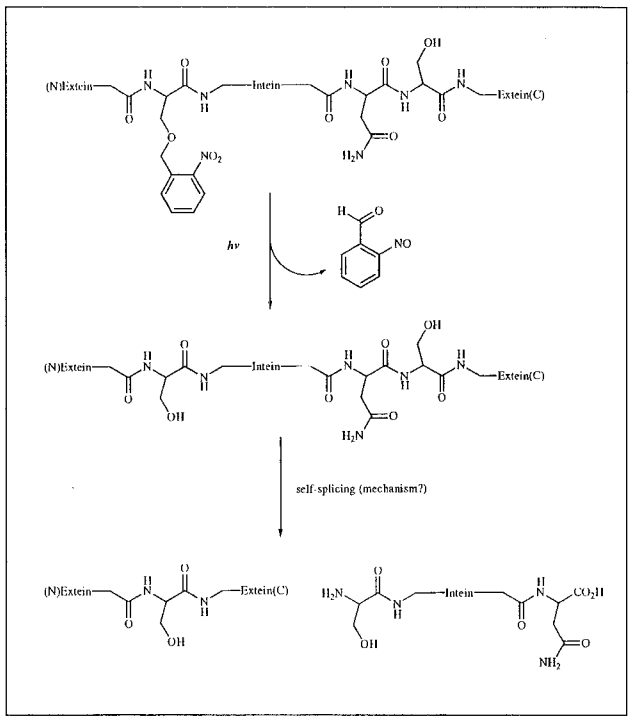


Fig. 3. Photoactivated self-splicing of Vent DNA polymerase using a caged serine analog. Analogous to exons and introns, an initial polypeptide is synthesized that contains both exteins and an intein; excision of the intein and ligation of the amino-terminal and carboxy-terminal exteins produces the mature, functional protein.

given the influence of context. In addition, the stabilities and simulated structures of the *t*-butylglycine mutants provide information about the effects of β-branched side chains on α-helix stability in a protein.

The results described in this section emphasize the difficulty of interpreting mutagenesis data when more than one property of an amino acid is being altered at once. The ability to precisely alter the structure of an amino acid should make it possible to obtain a better understanding of the individual contributions made by hydrophobicity, packing, entropy and cavity formation to protein stability. We are currently extending these studies to hydrogen bonding and π–π and π–ion interactions in protein interiors.

Enzyme mechanism

Unnatural amino acid mutagenesis is also being used to probe the catalytic mechanisms of the enzymes SNase, aspartate aminotransferase, methionine aminopeptidase and ribonucleotide reductase. SNase accelerates the hydrolysis of phosphodiester bonds in nucleic acids some 10^{16}-fold over the uncatalyzed rate. This enzyme has been the subject of many structural, mechanistic and mutagenesis studies aimed at understanding how enzymes can achieve such extraordinary rate enhancements [50–54]. On the basis of these studies it has been suggested that general base catalysis contributes significantly to the catalytic efficiency of this enzyme. Specifically, it is thought that Glu43 in SNase acts as a general base in activating a water molecule that attacks the phosphodiester backbone of DNA. Glu43 is known to be important for catalysis because replacement by the natural amino acids aspartate and glutamine results in a significant loss in activity [55]. Surprisingly, substitution of Glu43 with either homoglutamate **4** or the nitro analog *S*-4-nitro-2-aminobutyric acid **12**, which is both isoelectronic and isosteric to glutamate but a much poorer base, yields mutant enzymes with kinetic constants markedly similar to those of wild-type SNase under normal assay conditions (Fig. 4) [25•]. The catalytic efficiencies of these mutants, coupled with their pH behavior and the crystal structure of the homoglutamate 43 mutant, suggest that Glu43 may not be acting as a base, but may be structural, serving as a bidendate hydrogen-bond acceptor to fix the conformation of the neighboring loop. Independent studies by Gerlt and colleagues [56], in which the loop adjacent to Glu43 was deleted from SNase, have led to the same conclusion.

Cellular signal transduction

Unnatural amino acid mutagenesis has been used to probe the function of Ras p^{21} in cellular signal transduction pathways. Mammalian proteins encoded by the *ras* genes are thought to act as regulators of various signal transduction processes involved in cell growth and differentiation [57–59]. The chemical basis for signal regulation involves cycling of the protein between the inactive and the active GTP-bound states. Point mutations that decrease the intrinsic GTPase activity of Ras and the GTPase activity stimulated by the GTPase-activating protein are associated with approximately 30% of human cancers [58,60]. In order to gain a better understanding of the molecular basis by which mutations in Ras lead to switch inactivation, we substituted residues in loop L4 (the switch II region), loop L2 (the switch I region) and loop L1 (the phosphate binding loop) with a series of unnatural amino acids [61•,62•,63].

Mutations at Gly12 of Ras result in impaired intrinsic GTPase activity and are commonly associated with oncogenic activation [58,64]. Gly12 occurs in a highly conserved type II β-turn, a phosphate-binding loop found in many nucleotide-binding proteins [65,66]. Mutation of Gly12 to any common amino acid other than proline results in diminished GTPase activity [64]. To gain a better understanding of the effect of Gly12 mutants in switch function, we inserted a number of unnatural amino acids at this site, including lactic acid **9**, pipecolic acid **13** and *N*-methylglycine **14** [62•]. The pipecolic acid mutant, which was expected to have a much more negative ϕ value than Gly12, retained GTPase activity similar to that of wild-type Ras. The *N*-methylglycine mutant also had wild-type GTPase ac-

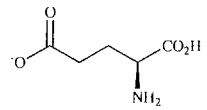
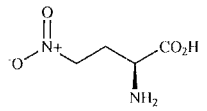
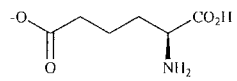
Amino Acid	Vmax (A_{260} min^{-1} μg^{-1})	K_M^{DNA} (μg/mL)	K_M^{Ca2+} (μM)
 glutamate	6.7 ± 0.7	8 ± 3	320 ± 30
 *S*-4-nitro-2-aminobutyric acid	3.0 ± 0.5	26 ± 8	470 ± 40
 homoglutamate	5.2 ± 0.2	10 ± 2	290 ± 30

Fig. 4. Catalytic efficiencies of wild-type staphylococcal nuclease and mutants Glu43→homoglutamate, and Glu43→*S*-4-nitro-2-aminobutyric acid.

tivity (N_α methyl substitution also decreases ϕ values). Whereas the Ala12 mutant had reduced GTPase activity, the isosteric lactic acid mutant, in which the backbone amide linkage is replaced by an ester linkage, had normal GTPase activity. As only those mutants that can adopt unusual backbone conformations are active, a conformational change in loop L1 may relieve unfavorable side chain–transition state interactions at position 12.

Even though the proline, pipecolic acid, lactic acid and *N*-methylglycine mutants at position 12 had intrinsic GTPase activity similar to that of wild-type Ras, they were not activated by GTPase-activating protein. Moreover, the Gly13→Thr, Gly13→*allo* Thr **15** and Gly13→Ser mutants, which have intrinsic GTPase activity two to three times that of wild-type Ras (attributable to the β hydroxyl group), were not activated by this activating protein and were not transforming in a germinal vesicle breakdown assay with *Xenopus laevis* oocytes [62•]. One interpretation of these results is that loop L1 can adopt two or more conformations in solution and that its positioning by one or more effector proteins modulates GTPase activity and oncogenic activation. The unusual backbone structures of the Gly12 and Gly13 mutants may prevent switch function.

In another mutagenesis experiment the role of Pro34 in loop L2 function was probed [61•]. The most significant difference between the GTP- and GDP-bound forms of Ras is in this region (residues 32–40) [67,68]. It has been proposed that Pro34, which is conserved in Ras and close to the active site, may assist in controlling the conformation of loop L2, perhaps via a *cis–trans* isomerization of the Pro34 amide bond [69]. In order to examine the structural and mechanistic function of this residue more precisely, Pro34 was replaced with 2,4-methanoproline **16**, which is strongly biased toward the *trans*-configuration by the C_α substitution. The fact that this mutant has wild-type intrinsic and protein-activated GTPase activity suggests strongly that a *cis–trans* isomerization of Pro34 is not of key importance in signal transduction. Similar substitutions may be useful in probing the role of backbone isomerization in protein folding pathways.

Conclusions

The ability to incorporate unnatural amino acids site-specifically into proteins makes it possible to carry out detailed physical organic studies on proteins. Mutations can be made to probe the precise nature of an individual amino acid's contribution to protein structure or function, often without dramatic losses in activity. This methodology should provide the opportunity to test assumptions about and improve our understanding of protein stability and enzyme catalysis. In addition, the technique may facilitate the generation of mutant proteins with novel, interesting properties.

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