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Research Overview

Substrate-Assisted Catalysis: Implications for Biotechnology and Drug Design

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ABSTRACT In enzyme catalysis, the convention is that the enzyme supplies all the functional groups that are needed to convert a substrate into a product. This convention, however, is now recognized to have some exceptions. In a growing number of cases it is evident that the substrate also provides one or more functional groups that actively participate in the catalytic process. These cases are grouped together under the title "substrate-assisted catalysis" (SAC). Examples of SAC have been described both for native and for engineered enzymes that were rendered inactive by mutations. Such mutations eliminate amino acid sidechains that participate in the catalytic process and thereby cause partial or total loss of enzymatic activity. For several of these mutant enzymes, a modified substrate bearing functional groups similar to those that had been eliminated by the mutation was found to rescue enzyme activity. A notable example is the mutual specificity found between mutant serine proteases and their modified substrates. This creates a highly specific site for proteolytic cleavage, a desirable property in the processing of recombinant fusion products. An attractive target for SAC is the G-protein family. It was applied to two of its members—Gsα and p21-Ras. In both cases it was possible to restore the GTPase activity of the mutants back to the level of the wild-type proteins. Beyond restoring activity by SAC, further modifications of the substrate were introduced to support or refute particular roles of the functional groups in the GTPase reaction. This approach was also applied as a molecular tool to discriminate between specific enzymatic mechanisms and as a guideline to incorporate particular functional groups into the substrate. Taken together, these studies pave the way to novel therapeutic and biotechnological approaches aimed at restoring the activity of mutant inactive enzymes. Drug Dev. Res. 50:250–257, 2000.

Key words: substrate-assisted catalysis; G-proteins; Ras; serine proteases; restriction endonucleases

INTRODUCTION

Enzymes perform their catalytic function by various molecular mechanisms. Understanding the intricate mechanisms by which enzymes exert their catalytic power has been a major effort in biochemical research for many years. In general, the enzymatic process can be described as lowering the activation energy of the enzyme-catalyzed reaction by preferential stabilization of the transition state. All enzymes bind their substrates noncovalently as a prerequisite to actual catalysis. In addition, some examples of enzymatic mechanisms involve a transient covalent bond between the substrate and a specific residue in the enzyme. In this context, the prevalent view of enzymatic catalysis is that an enzyme acts on its substrates without the active participation of the substrates in the actual catalytic process. Remarkably, however, some examples

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of specific enzymatic reactions are an exception to this convention. In these cases, the substrate provides a functional group that actively participates in the catalytic process. It resembles examples of intramolecular catalysis in organic chemistry, where proximally located functional groups of a reactant are involved in the catalysis of a chemical reaction [Fersht, 1985]. An enzymatic reaction in which a functional group of the substrate acts cooperatively with the functional groups of the enzyme to achieve catalysis has been termed "substrate-assisted catalysis" (SAC). SAC of enzymatic reactions is a novel concept that has not been appreciated until relatively recently. Examples of SAC are found both in naturally occurring enzymes and in artificially engineered enzyme-substrate pairs. For an up-to-date general description of SAC, readers are referred to the only review on this topic [Dall'Acqua and Carter, 2000]. In the present review, we concentrate mainly on engineered SAC, as it is highly relevant to biotechnology and drug development. In these fields it can be used as a probe to unravel the mechanism of enzyme action, to define substrate specificity, identify drug targets, and as a novel processing tool for recombinant proteins. It can also be the first step toward restoring activity to enzymes whose catalytic ability has been impaired by mutation.

USING SAC AS A MOLECULAR PROBE

In the last decades, the understanding of enzymes has grown immensely as their structure has been determined using X-ray crystallography and NMR. Despite the vast knowledge accumulated to date, information about the exact contribution to catalysis by specific residues or the dynamics of the enzymatic reactions is insufficient at best. In a complementary approach, SAC can be used as a molecular analytical tool to answer such questions in intricate detail. It is particularly useful in cases where an enzyme has been inactivated by mutation and structure–function analysis is difficult to achieve by conventional methods. Such point mutations, which result in a substantial loss of catalytic activity, suggest that the amino acid that has been mutated participates in the catalytic process. This provisional functional assignment must be further corroborated by structural information and mechanistic consideration. When this is achieved, the question still remains as to what part of the loss in catalytic activity is due to functional effect and what part is due to structural changes. Even more important, from a practical point of view, is the question whether the loss of activity is due to a reversible or irreversible change in the enzyme. Application of SAC not only gives definite answers to these questions, but can also restore activity to the mutant inactive enzyme (see Fig. 1). Such an approach has been most successfully applied to guanine nucleotide binding proteins. Since application of SAC to mutant G-proteins resulted in the most efficient example of restoring activity to a mutant enzyme up to the level of the wild-type protein, this will be described in detail below.

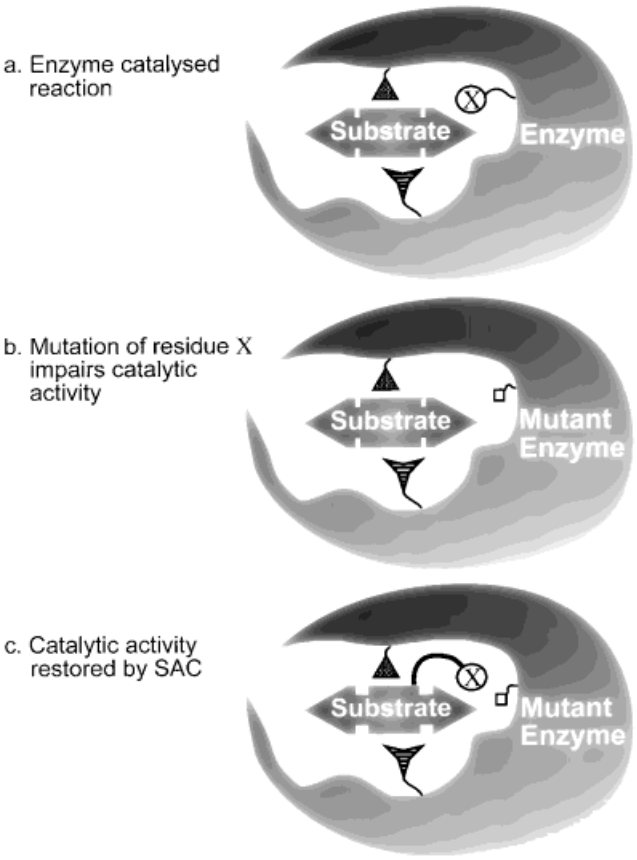

Fig. 1. The concept of substrate-assisted catalysis: **a.** enzymatic catalysis, the conversion of a substrate into product, is dependent on several functional groups in the active site of the enzyme. **b.** When a residue bearing a catalytic functional group (marked X) is eliminated by mutation, catalytic activity is impaired. **c.** Placing the missing functional group correctly in the mutated active site restores catalytic activity. In SAC, this functional group is brought into the active site by a modified substrate.

SAC IN THE CATALYTIC MECHANISM OF RESTRICTION ENDONUCLEASES

Type II restriction endonucleases are part of the daily tools of molecular biologists. This large family of enzymes encompasses thousands of members, with hundreds of different substrate specificities. They recognize a specific palindromic sequence in double-stranded DNA and cleave both strands, with the products having 3′ hydroxyl and 5′ phosphate ends [Pingoud and Jeltsch, 1997]. Although the catalytic mechanism of endonucleases has not been fully elucidated, evidence has accumulated to implicate SAC in this naturally occurring enzymatic reaction [Dall'Acqua and Carter, 2000].

Hydrolysis of the phosphodiester bond apparently proceeds with an attack of a water molecule, in-line to the 3′ hydroxyl leaving group. Structure analysis led to the hypothesis that the phosphate group, immediately 3′ to the bond hydrolyzed, acts as a general base to activate the attacking nucleophilic water molecule [Jeltsch et al., 1992, 1993]. A similar hypothesis was raised for the mechanism of GTP hydrolysis in G-proteins, as detailed below. This involvement of SAC in activation of the nucleophile in endonucleases was investigated in the most logical manner—using modified substrates to test different roles for the involvement of the proximal phosphate. Substrates lacking a negative charge at this position were cleaved very slowly or not at all [Jeltsch et al., 1993]. On the other hand, modifications capable of deprotonating the attacking water molecule could substitute for the native phosphate, supporting the role of the phosphate as a general base [Koziolkiewicz and Stec, 1992; Thorogood et al., 1996].

Although more research is needed to reach complete understanding of the reaction's mechanism, it seems highly probable that the substrate for endonucleases takes an active catalytic part in its own hydrolysis. It appears that nature came up with the concept of SAC long before researchers thought of the same approach. In the next sections, however, we will elaborate on how SAC was engineered a step further, extending beyond natural evolution.

ENGINEERED SAC — SERINE PROTEASES

Serine proteases are some of the most extensively studied families of enzymes. For this group of enzymes, both structural and functional information is available in exquisite detail. Despite differences in substrate specificity and in the range of the accommodated substrates, serine proteases employ a common triad of catalytic amino acids to accomplish catalysis: serine, histidine and aspartate. The histidine residue has been suggested to play a dual role in the two-step catalysis by serine proteases. In the first step, acyl-enzyme formation, histidine serves as a general base (proton acceptor), while in the second deacylation step, it serves as a general acid (proton donor) [Kraut, 1977].

The first demonstration of SAC in an engineered, mutant enzyme was with the subtilisin mutant in the catalytic histidine—H64A [Carter and Wells, 1987]. This mutation decreases catalytic activity by almost six orders of magnitude. Remarkable restoration of catalytic activity to subtilisin H64A was achieved by presenting the enzyme with substrates containing histidine in the P2 position [nomenclature in Schechter and Berger, 1968]. It was in these ground-breaking experiments that the term substrate-assisted catalysis was coined. While activity of the H64A subtilisin on a histidine-containing substrate was not restored to the level of the wild-type enzyme, the H64A subtilisin displayed an enhanced specificity for histidine-containing substrates in contrast to the broad specificity of the wild-type subtilisin enzyme. Considerable improvement of the catalytic efficiency of H64A subtilisin was achieved by optimizing both the enzyme and the histidine-containing substrates [Carter et al., 1989, 1991]. This efficient variant of H64A subtilisin was used for site-specific proteolysis of recombinant fusion protein products [Carter et al., 1989; Forsberg et al., 1991, 1992]. The principles of SAC have been extended to two other proteolytic enzymes, namely trypsin and elastase. These enzymes also use the aforementioned histidine, serine, and aspartate triad for catalysis. As expected, these enzymes also demonstrated remarkable reduction in catalytic activity upon mutation of the catalytic histidine to alanine. In the case of the H57A mutant, trypsin activity was partially restored by substrates containing histidine either in the P1 or P2 sites. Substrates containing histidines in both the P1 and P2 sites further increased the catalytic efficiency by about fourfold [Corey et al., 1995]. In the case of H57A elastase, a phage display library of randomized peptides was screened for peptides that would restore activity to the mutant elastase. All of the efficient substrates contained histidine in the P2 site [Dall'Acqua et al., 1999]. Remarkably, the phage derived sequence REHVVY was cleaved by H57A elastase at a rate that was only 160-fold slower than the rate of cleavage by the wild-type enzyme. Taken together, the studies on serine proteases not only introduced and created the concept of SAC but also established a useful biotechnological application using a mutant enzyme and a tailor-made substrate. These can be combined to create a highly specific tool for protein processing and cleavage, both for basic research and for biotechnology.

GUANINE NUCLEOTIDE BINDING PROTEINS — A HIGHLY CONSERVED FAMILY

Guanine nucleotide-binding proteins (referred to as G-proteins or GTPases) are transducers of a wide range of essential cellular transactions. Transmembrane signaling, cell proliferation, intracellular trafficking, and cytoskeletal organization are notable examples. Despite this functional diversity, all G-proteins behave as conformational sensors with respect to the identity of the guanine nucleotide that is bound in their active site. Dependent on whether they are charged with GDP or GTP, they change their conformation and consequently their interaction with other signaling proteins. All G-proteins, when charged with GTP, are in the "on" state, while G-proteins that are charged with GDP are in the "off" state. A common regulatory switch mechanism (the regulatory GTPase cycle, see Fig. 2) controls the downstream

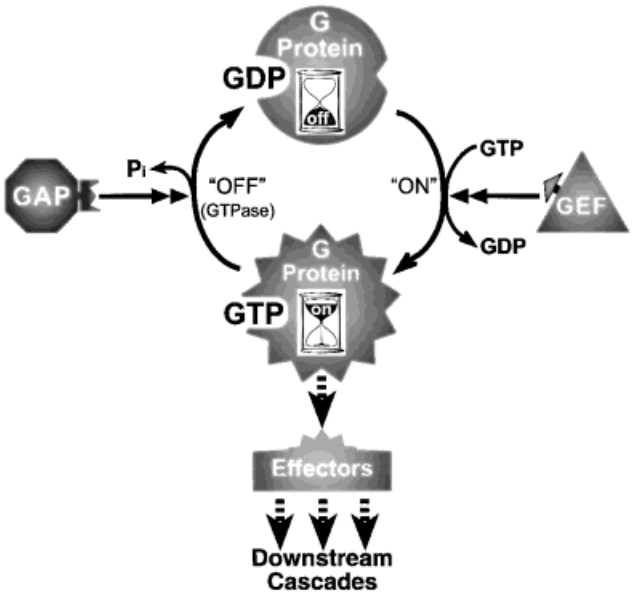

Fig. 2. The regulatory GTPase cycle: a G-protein functions as a molecular switch. It is held in the "off" position by GDP, which is tightly bound to its active site. Exchange of the bound GDP for GTP turns the G-protein to the "on" position. This reaction is enhanced by guanine nucleotide exchange factors (GEFs) specific to each G-protein. When it is turned "on," the G-protein interacts and activates downstream signaling cascades through its various effectors. The downstream activity of the G-protein is controlled by an internal timer – the intrinsic GTPase reaction that gives rise to free inorganic phosphate and tightly bound GDP. This process of turning "off" of the internal timer can be accelerated by orders of magnitudes of GTPase Activating Proteins (GAPs). Conversely, impairment of the GTPase reaction will in effect slow down the timer. The result will be excess G-protein in the "on" state and hyperactivation of the downstream cascades. This can lead to abnormal and sometimes pathological phenotypes.

activity of the G-proteins [Cassel et al., 1977]. It is based on a catalytic hydrolysis of GTP with a K_{cat} and turnover number that can be increased by several orders of magnitude upon interaction with other signaling molecules. A unique feature of this GTPase reaction is that the GDP product of the reaction does not dissociate from the protein and its tight binding stabilizes the G-protein in the inactive state. To activate the G-protein, the bound GDP must be released and replaced by free GTP, which is the prominent guanine nucleotide in the cytosol.

This checkpoint reaction is greatly facilitated by guanine nucleotide exchange factors (GEFs), each specific for a particular G-protein. Another regulatory checkpoint is the hydrolysis of the bound GTP. The intrinsic GTPase reaction is rather slow, but can be accelerated by several orders of magnitude upon interaction with GTPase activating proteins (GAPs). This pivotal GTPase reaction was first described 25 years ago [Cassel and Selinger, 1976] and since then has attracted considerable attention because it determines the lifetime of the active G-protein. Any disruption of the GTPase reaction results in a persistent activation of the downstream effectors since the G-protein is not switched off. Such a situation is easily recognized as a gain of function mutation at the effector level, although the primary effect is a loss of function at the level of the GTPase reaction. Several GTPase deficient G-protein mutants, mainly in Ras and $G_{s\alpha}$, have been implicated in tumor formation [Bos, 1989; Dhanasekaran et al., 1998; Lyons et al., 1990; Spiegel, 1996; Vallar, 1996]. In particular, the GTPase reaction of Ras is of great medical importance because about 30% of all human tumors contain mutations in Ras, which result in an impaired GTPase activity. Furthermore, these oncogenic mutants can not be switched off by GAPs. Consequently, the GTPase deficient G-proteins persistently drive cell division and contribute substantially to the neoplastic phenotype of tumor cells. This has given rise to the idea that restoring the GTPase activity of oncogenic Ras mutants might be a useful therapeutic approach to cancer.

EXPLORATION AND RESCUE OF THE GTPASE REACTION IN $G_{s\alpha}$

The first indication that the GTPase reaction is in fact a turn-off mechanism came when cholera toxin was found to inhibit the GTPase reaction of the adenylyl cyclase stimulatory G-protein ($G_{s\alpha}$) and thereby caused persistent activation of cyclic AMP production [Cassel and Selinger, 1977]. The inhibition of GTP hydrolysis by cholera toxin was shown to be due to ADP-ribosylation of Arg201 in the stimulatory G-protein $G_{s\alpha}$ subunit.

Mutations of either Arg201 or Gln227 in $G_{s\alpha}$ have been observed in thyroid and pituitary tumors [Landis et al., 1989; Lyons et al., 1990]. In accord, site-directed mutagenesis of either Arg201 or Gln227 in $G_{s\alpha}$ was found to inhibit its GTPase activity in vitro [Graziano and Gilman, 1989; Landis et al., 1989]. While these studies pointed to the importance of the conserved Gln227 and Arg201 in GTP hydrolysis by $G_{s\alpha}$, a fundamental breakthrough came from the determination of the three-dimensional structure of the α subunits of two heterotrimeric G-proteins: transducin ($G_{t\alpha}$) [Lambright et al., 1994] and the adenylyl cyclase inhibitory G-protein ($G_{i\alpha1}$) [Mixon et al., 1995]. In these studies, the structure of the G-protein α-subunits was determined with the substrate analog guanosine 5′-[γ-thio] triphosphate (GTPγS) and with the product (GDP). However, even more important to the understanding of the GTPase mechanism was the determination of the structure of both $G_{t\alpha}$ and $G_{i\alpha1}$ in complex with aluminum fluoride (AlF^-_4) [Coleman et al., 1994; Sondek et al., 1994]. Initially, it was suggested that the aluminum occupies the position of the γ-phosphate of GTP [Bigay and Deterre, 1985; Higashijima et al., 1991]. This notion was based on the tendency of AlF^-_4 to form coordinated bonds with phosphate. Surprisingly, structural analysis of these complexes revealed that they do not resemble the structure

of the G-protein with its substrate. Rather, these structures show the presence of a GDP-AlF^-_4-water complex, which resembles in its geometry the structure of the presumed pentacovalent transition state of the GTPase reaction. In both structures, the GDP-AlF^-_4 complex is stabilized by interactions with the catalytic glutamine and arginine. These studies and others strongly support an associative mechanism for the GTPase reaction that includes an in-line attack by a water molecule. Despite the exquisite information which has been provided by these groundbreaking studies, it is not clear how the GTPase reaction proceeds, what is the identity of the general base that accepts the proton from the attacking water molecule, and how the glutamine residue participates in this reaction. Indeed, some of these questions are difficult to answer by structural analysis. SAC was applied to the stimulatory G_s protein of the adenylyl cyclase system to test the role of the glutamine in the GTPase reaction.

To this end, mono- and di-aminobenzophenone phosphoroamidates-GTP (MABP and DABP-GTP, respectively, see Fig. 3) were synthesized and tested in the adenylyl cyclase system [Zor et al., 1997]. The GTP analog DABP-GTP has both the amino and the carbonyl groups of glutamine, whereas MABP-GTP has only the carbonyl group.

It was observed that the analog DABP-GTP is hydrolyzed by G_s at the same rate as GTP, whereas MABP-GTP, lacking the free aromatic amino group, was found to be hydrolysis-resistant. Furthermore, DABP-GTP was found to rescue the GTPase activity of the mutant Gln227Leu G_s back to the level of the wild-type G_s. In contrast, DABP-GTP was unable to accelerate GTP hydrolysis by the GTPase deficient G_s, in which the catalytic arginine is ADP-ribosylated by cholera toxin. This latter finding attests to the specificity of DABP-GTP, as it rescues the catalytic function of glutamine but not of arginine.

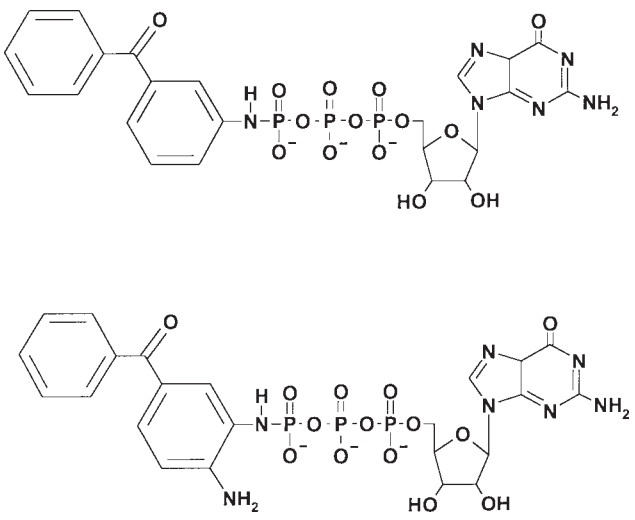

Fig. 3. MABP-GTP: 3-mono-amino-benzophenone-phosphoroamidate GTP. DABP-GTP: 3,4-di-amino-benzophenone-phosphoroamidate GTP.

These studies were followed by experiments to test the role of glutamine in the GTPase reaction of G_s. Once it was established that the carbonyl group is not needed for hydrolysis of the GTP analog, a series of GTP analogs carrying different substituents on a benzene ring were synthesized. Their resistance or susceptibility to hydrolysis by $G_{s\alpha}$ elucidated the functional requirements for the analog hydrolysis [Zor et al., 1998]. It was concluded that for efficient catalysis a hydrogen donor group must be present in close proximity to the γ-phosphate of GTP. In the hydrolysis of GTP, this function is probably carried out by the catalytic glutamine. On the other hand, in the hydrolysis of the GTP analogs this function is fulfilled by modifications which bear either a free amino or a hydroxyl group in an ortho position to the phosphoroamidate linkage. Interestingly, nature has utilized a similar variation in the small G-protein Rap. In this G-protein, the conserved catalytic glutamine is substituted by a threonine. Nevertheless, Rap hydrolyzes GTP quite efficiently in the presence of Rap GAP1 [Chen et al., 1997]. Thus, the conclusions drawn by the use of SAC to unravel the functional requirements of enzyme catalysis have in fact rediscovered what had been evolved by nature long ago. This validates the use of SAC for structure–function analysis of enzymatic reactions.

SAC IN NATIVE RAS

Ras is a small G-protein that regulates cell proliferation and differentiation in response to extracellular growth factors. As in all the G-protein mediated transduction pathways [Cassel et al., 1977], signal flow is controlled by the function of Ras as a molecular switch, cycling between a GDP-bound inactive state and a GTP-bound active state (Fig. 2). Similarly, the relative levels of the active and inactive Ras in the cell are determined by the activities of guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). The GEFs facilitate GDP release, which is followed by binding of GTP and hence activate Ras. GAPs greatly increase the slow intrinsic GTPase reaction of Ras and thereby inactivate it [Scheffzek et al., 1998; Gamblin and Smerdon, 1998].

A fundamental breakthrough in understanding the mechanism of the GTPase reaction in Ras was provided by determination of the three-dimensional structures of Ras with Ras-GAP using X-ray crystallography [Scheffzek et al., 1997]. This study and previous work corroborated the suggestion that GTP hydrolysis is carried out by a nucleophilic attack of a water molecule. This nucleophilic water is present in close proximity to the γ-phosphate and to the sidechain of Gln61. This spatial arrangement and the findings that mutations in Gln61 dramatically decrease the rate of the GTPase reaction [Der et al., 1986]

led to the suggestion that Gln61 participates in hydrolysis of GTP by functioning as a general base. However, both theoretical [Langen et al., 1992] and experimental analysis [Schweins et al., 1994] did not support this hypothesis. A short time later, a combination of in-depth theoretical considerations and elegant experiments largely resolved the problem of what is the elusive general base in the GTPase reaction of Ras [Schweins et al., 1995, 1996; Schweins and Warshel, 1996]. These studies suggest that in the Ras GTPase reaction the substrate itself (GTP) acts as the catalytic base in a mechanism of SAC. Furthermore, the close similarity between the active sites of $G_{i\alpha 1}$ and Ras in the transition state [Coleman et al., 1994; Scheffzek et al., 1997] makes it likely that a mechanism of SAC in which the substrate GTP serves as a general base holds true for G-proteins in general.

RESCUE OF GTPASE ACTIVITY BY SAC IN ONCOGENIC RAS MUTANTS

Interest in the GTPase reaction of Ras stems from the observation that in a large number of human tumors Ras is mutated at amino acid positions 12 or 61, and more rarely at position 13. These mutant Ras proteins are found in about 30% of all human tumors, including some of the most vicious cancers. All of these oncogenic mutant Ras proteins have defective GTPase activity and can no longer be switched off by GAPs. As a result of their impaired GTPase reaction, oncogenic Ras proteins are not switched off, they persistently drive cell division and contribute substantially to the neoplastic phenotype of tumors.

Despite the fact that Ras has been in the focus of drug discovery programs for quite some time, no compound consistently increasing the GTPase activity of oncogenic Ras proteins has been reported so far. Application of SAC to oncogenic Ras proteins has been used to find the answers for three questions: 1) Are the mutant Ras proteins irreversibly damaged? 2) Can the oncogenic Ras protein be rescued when presented with suitable chemical groups in the appropriate geometry? 3) Is the blocked GTPase reaction a justified target for anticancer drug development? Application of DABP-GTP (Fig. 3) to the Gln61Ala mutant of Ras showed that the GTP analog is hydrolyzed at a rate which is 180 times faster than the rate of GTP hydrolysis. In fact, this Gln61 mutant hydrolyzed DABP-GTP at the same rate as the wild-type Ras. This indicated that Gln61 is not required for hydrolysis of DABP-GTP. Unexpectedly, the hydrolysis of DABP-GTP was dramatically enhanced with the Gly12 mutants, which are the most common mutations in human tumors [Bos, 1989; Der et al., 1986]. The rate of the intrinsic GTPase activity of the Gly12Val mutant is 0.0024 min^{-1} at 37°C while the rate of DABP-GTP hydrolysis is 3.1 min^{-1} [Ahmadian et al., 1999]. This is a 720-fold increase as compared with the GTPase of the mutant and 110-fold increase with respect to wild-type Ras. In general, the hydrolysis rate of the analog by all the Gly12 mutants tested was dramatically and selectively increased, as the DABP-GTPase rates of the mutants were much faster than both the GTPase and DABP-GTPase of wild-type. Rescue of the catalytic properties of this Ras mutant is not only independent of the presence of Gln61 but is actually more efficient in its absence. This was found by experiments using the double mutants V12A61-Ras and V12L61-Ras, which showed about a 1,000-fold rate increase as compared with the rate of GTP hydrolysis. These findings raised the following question: What is the underlying mechanism that explains both the rescue of the catalytic activity of the mutant Ras by DABP-GTP and the dramatic increase of the GTP-analog hydrolysis over the hydrolysis of GTP? To answer this question the rate of GTP and DABP-GTP hydrolysis was measured in H_2O and deuterium oxide (D_2O). These experiments test whether proton transfer is the rate-limiting step in the reaction. If this is the case, one expects to see an isotope effect causing a decrease in the rate of the reaction in D_2O over the rate in H_2O. As noted before for the Ras-mediated GTP hydrolysis, the γ-phosphate of GTP abstracts a proton from the nucleophilic water molecule [Schweins et al., 1995]. It has also been found, however, that this is not the rate-limiting step, since a solvent isotope effect was not found for the overall reaction of GTP hydrolysis. In contrast, a strong isotope effect was observed using DABP-GTP as a substrate. A twofold reduction in the rate of the reaction in D_2O over the rate of the reaction in H_2O was found [Ahmadian et al., 1999]. It was argued that in the hydrolysis of DABP-GTP the rate-limiting step of the GTPase reaction is bypassed and proton transfer became the rate-limiting step of DABP-GTP hydrolysis. The following mechanism can explain these results: the rate-limiting step of the GTPase reaction is the positioning of the glutamine in such a location where it can interact with the attacking water molecule. With DABP-GTP, the aromatic amine presumably substitutes for the catalytic glutamine. This amino group is in a fixed position, close enough to interact with the water molecule and thereby DABP-GTP bypasses the rate-limiting step of the GTPase reaction. Taken together, these results show that the oncogenic phenotype of Ras mutants can be inactivated chemically and that they are not irreversibly damaged in their capacity to act as molecular switches. In principle, it should be possible to find compounds that incorporate the relevant chemistry and, at the same time, bind with sufficient affinity to the active site of Ras. Such a compound would not be expected to interfere with the GTPase reaction of wild-type Ras because the latter is downregulated much more efficiently by GAPs. Therefore, it is expected to have fewer side effects than current anti-Ras drugs.

CONCLUSIONS

It is becoming evident that SAC is more frequently used in enzyme action than has previously been observed. Apparently, nature has utilized SAC in the evolution of enzymatic machinery long before biochemical research came upon this concept. Well-planned research, however, took SAC a step further, making it a tool both for analytical and applied investigations. Changing the specificity and type of enzymatic activities can be most useful in creating new applications for enzymes in biotechnology. SAC can answer questions that are difficult to cope with using other approaches. Furthermore, this molecular tool can solve specific problems in bioengineering and in drug design. Using SAC in mutant, inactive enzymes is the first step on the road for rational design of drugs aimed at restoring activity to these damaged enzymes.

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