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**REVIEW**

# Substrate-assisted catalysis: Molecular basis and biological significance

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**Abstract**

Substrate-assisted catalysis (SAC) is the process by which a functional group in a substrate contributes to catalysis by an enzyme. SAC has been demonstrated for representatives of three major enzyme classes: serine proteases, GTPases, and type II restriction endonucleases, as well as lysozyme and hexose-1-phosphate uridylyltransferase. Moreover, structure-based predictions of SAC have been made for many additional enzymes. Examples of SAC include both naturally occurring enzymes such as type II restriction endonucleases as well as engineered enzymes including serine proteases. In the latter case, a functional group from a substrate can substitute for a catalytic residue replaced by site-directed mutagenesis. From a protein engineering perspective, SAC provides a strategy for drastically changing enzyme substrate specificity or even the reaction catalyzed. From a biological viewpoint, SAC contributes significantly to the activity of some enzymes and may represent a functional intermediate in the evolution of catalysis. This review focuses on advances in engineering enzyme specificity and activity by SAC, together with the biological significance of this phenomenon.

**Keywords:** catalytic mechanism; neighboring group participation; substrate-assisted catalysis

The concept of intramolecular catalysis, in which a reaction is accelerated by the participation of a neighboring group in a reactant, is well established in organic chemistry (reviewed by Fersht, 1985). In contrast, the role of intramolecular catalysis in enzymes by substrate participation—"substrate-assisted catalysis" (SAC)—has only recently started to be appreciated.

Enzyme-substrate pairs are typically predicted to be candidates for SAC if the substrate contains a functional group that can participate in catalysis in a way that is plausible from mechanistic, structural, and stereochemical standpoints. If SAC is indeed occurring, then removal of the relevant functional group from the substrate should impair catalysis. In some cases, additional evidence for SAC has come from the pH dependence of catalysis, mutational analysis of the enzyme, and linear free energy relationships (Table 1).

SAC has been convincingly demonstrated in the context of several engineered enzymes including representatives of two major classes, serine proteases and GTPases, as well as lysozyme and hexose-1-phosphate uridylyl transferase (Table 1). SAC also contributes to the activity of naturally occurring enzymes as evidenced by members of two large classes, GTPases and type II restriction endonucleases, and additionally lysozyme (Table 1). Proposed mechanistic models for SAC (Fig. 1) await experimental testing and refinement. Several additional examples of SAC predicted from structural data, remain to be evaluated through structure-function analysis, and are presented in summary form (Table 1). Here, we present evidence for SAC in natural and engineered enzymes along with the biological implications of this phenomenon.

## *Serine proteases*

Serine proteases contain a triad of catalytic residues comprising a serine, histidine, and aspartate. The catalytic histidine serves two roles in the hydrolysis of peptide bonds, acting first as a proton acceptor in the formation of an acyl enzyme intermediate and then as a proton donor in the subsequent deacylation step (Kraut, 1977). The critical role of the catalytic histidine in the serine protease, subtilisin BPN′, has been demonstrated by its replacement with alanine, which lowers the catalytic efficiency by approximately a million-fold (Carter & Wells, 1988).

### *Subtilisin: SAC with P2 and P1′ histidines*

The earliest demonstration of SAC with an engineered enzyme was with subtilisin BPN′ (Carter & Wells, 1987). A histidine at the P2 position of a substrate (Schechter & Berger, 1968) can be modeled such that the imidazoyl nitrogens virtually superimpose

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upon the corresponding nitrogens of the catalytic histidine, H64 (Carter & Wells, 1987). This observation led to the discovery that a P2 substrate histidine can substitute functionally for the missing catalytic histidine in the H64A subtilisin (Carter & Wells, 1987). The H64A enzyme has a strong preference for histidine-containing substrates as judged by a ~200-fold greater catalytic efficiency ($k_{cat}/K_m$) for succinyl-FAHF-*p*-nitroanilide than for related substrates containing alanine or glutamine in place of histidine. Moreover, the H64 enzyme was found to cleave some peptide substrates in a histidine-dependent manner. Thus, the H64A mutation converts subtilisin from a broadly specific to a histidine-specific protease, albeit at the cost of catalytic efficiency that is ~5,000-fold lower than for the wild-type enzyme. Subsequently, it was found that a P1′ histidine can also partially restore the function of the missing catalytic histidine in H64A subtilisin (Carter et al., 1989, Matthews & Wells, 1993). Indeed, the most favorable P1′ substrate is cleaved at rates comparable to the best P2 substrates.

*SAC mechanism and substrate binding by H64A subtilisin*

Several lines of evidence suggest that substrate binding by H64A subtilisin and the mechanism of SAC with a P2 histidine are qualitatively similar to substrate binding and catalysis by the wild-type enzyme (Carter et al., 1991). First, the formation of an acyl enzyme intermediate by H64A subtilisin (Fig. 1A) is suggested by the similar ratios of aminolysis to hydrolysis products for ester and amide substrates hydrolyzed in the presence of a dipeptide nucleophile. In contrast, the aminolysis to hydrolysis ratio would be expected to vary with leaving group ability if H64A subtilisin utilized a single-step mechanism (Fastrez & Fersht, 1973). Second, SAC is substantially impaired by mutation of the catalytic triad residues, S221 and D32, and also the oxyanion–stabilizing residue, N155 (Carter et al., 1991). These residues are also important to catalysis by wild-type subtilisin (Bryan et al., 1986; Wells et al., 1986; Carter & Wells, 1988). Third, mutation of any of these catalytic residues in H64A subtilisin abolishes the preference for

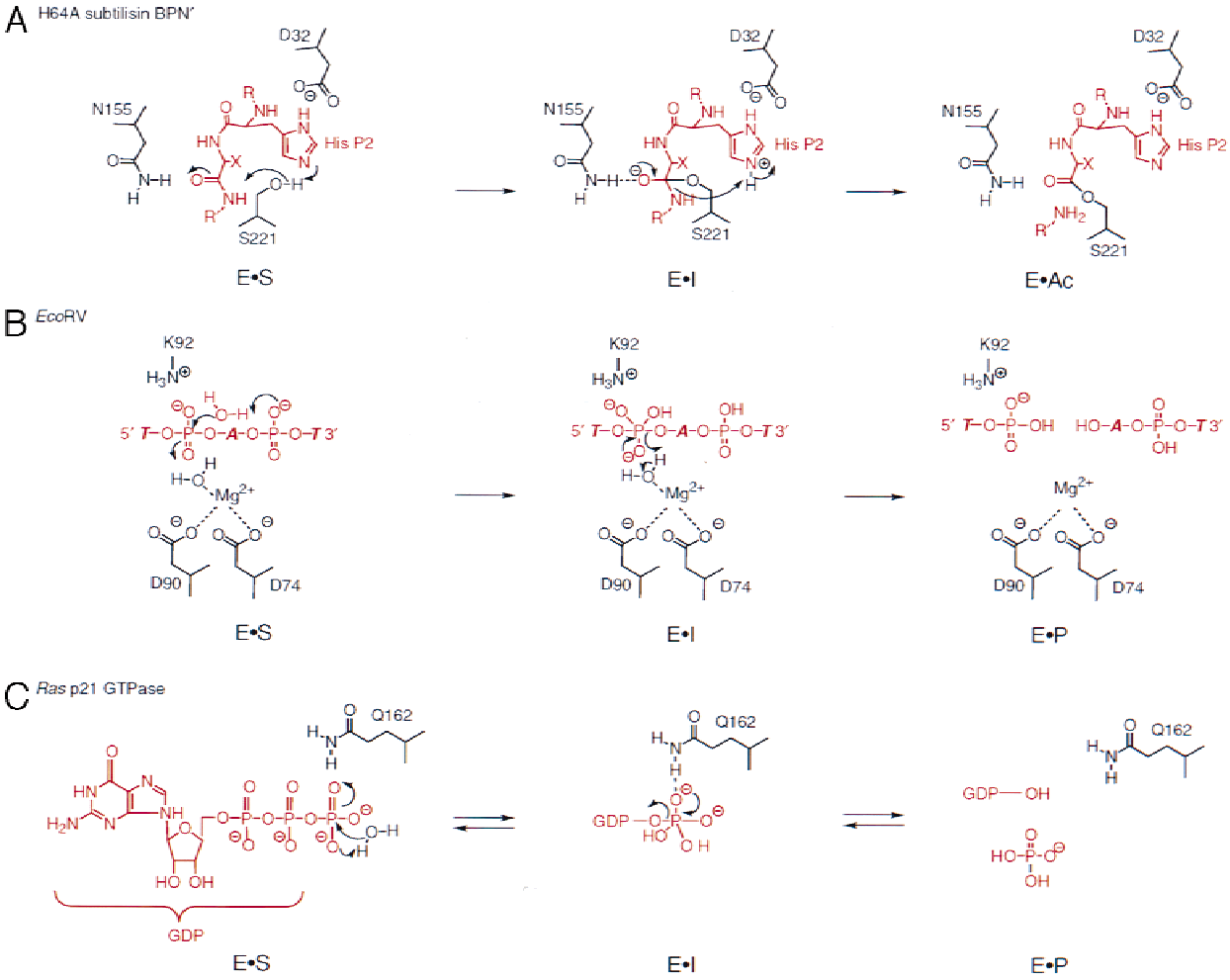


**Fig. 1.** Proposed mechanisms for SAC by (**A**) H64A subtilisin BPN′ with a peptide substrate containing a P2 histidine; (**B**) *Eco*RV with a GATATC double-stranded DNA substrate (TAT portion of one strand shown); (**C**) *Ras* p21 GTPase with GTP substrate; (**D**) Q227L $G_s\alpha$ GTPase with DABP-GTP substrate; (**E**) wild-type goose and D52A chicken egg white lysozymes with carboxymethyl chitin substrate; (**F**) H166G hexose-1-phosphate uridylyltransferase with glucose-1-phosphate (or galactose-1-phosphate) and uridine 5′-(phosphoimidazolate). Shown are enzyme-substrate (E·S), enzyme-intermediate (E·I), and enzyme-product (E·P) complexes. For engineered enzymes, the position of mutated catalytic residues emulated by the substrate has been omitted for clarity. In the case of subtilisin, an acyl enzyme intermediate (E·Ac) is formed. Subsequent deacylation is the reverse of the acylation reaction (shown), where water occupies the position of the leaving group amine. (*Figure continues on facing page.*)

histidine over glutamine at the P2 position (Carter et al., 1991). This is reminiscent of wild-type subtilisin where the catalytic function of H64 is critically dependent upon the presence of S221 and D32 (Carter & Wells, 1988). Fourth, the pH dependence of SAC by H64A subtilisin is very similar to that for the wild-type enzyme (Carter & Wells, 1987). This suggests that a deprotonated P2 histidine is important in catalysis by H64A subtilisin as is the case for H64 in the wild-type enzyme. Finally, the P1 and P4 subsite specificities are similar for SAC by H64A subtilisin as for the wild-type enzyme (see below).

Molecular modeling with subtilisin suggests significant differences in the mechanism of SAC depending upon whether a P2 or P1′ substrate histidine is utilized. Histidines can be modeled at both P2 and P1′ positions to form a hydrogen bond with the catalytic serine (Matthews & Wells, 1993), whereas only the P2 histidine is close enough to the catalytic aspartate to make a plausible favorable interaction. Moreover, a P1′ histidine may potentially participate in the anticipated acylation reaction, but is not available to participate in the presumed deacylation reaction. Thus, SAC by subtilisin with a P1′ histidine likely involves a catalytic dyad between the enzyme serine, S221, and the substrate histidine.

*Enhancing the efficiency of SAC*

The catalytic efficiency ($k_{cat}/K_m$) of H64A subtilisin has been increased ~160-fold to ~$10^4$ $s^{-1}$ $M^{-1}$ by cooptimization of the enzyme and histidine-containing substrate (Table 2) (Carter et al., 1989, 1991). Substrate optimization for H64A subtilisin was guided by detailed knowledge of the substrate specificity of the wild-type enzyme. In contrast, the H64A enzyme was optimized by recruitment of active site residues from other subtilisins (Carter et al., 1991) as well as by incorporation of mutations known to increase the activity of the wild-type enzyme. The identification of preferred protease substrates can be accomplished much more rapidly and thoroughly since the advent of substrate phage (Matthews & Wells, 1993) and combinatorial substrate libraries (Thornberry et al., 1997). Indeed, substrate phage have been used to identify favorable substrates for an enhanced activity variant of H64A subtilisin (Matthews & Wells, 1993). DNA shuffling (Stemmer, 1994) is a powerful molecular evolution technology (reviewed by Patten et al., 1997) that has been used for the rapid creation of enhanced activity and stability variants of subtilisin (Ness et al., 1999). DNA shuffling has great potential for evolving subtilisin and other enzymes for increased efficiency of SAC. In addition, two elegant strategies have recently been described for the selection of phage display

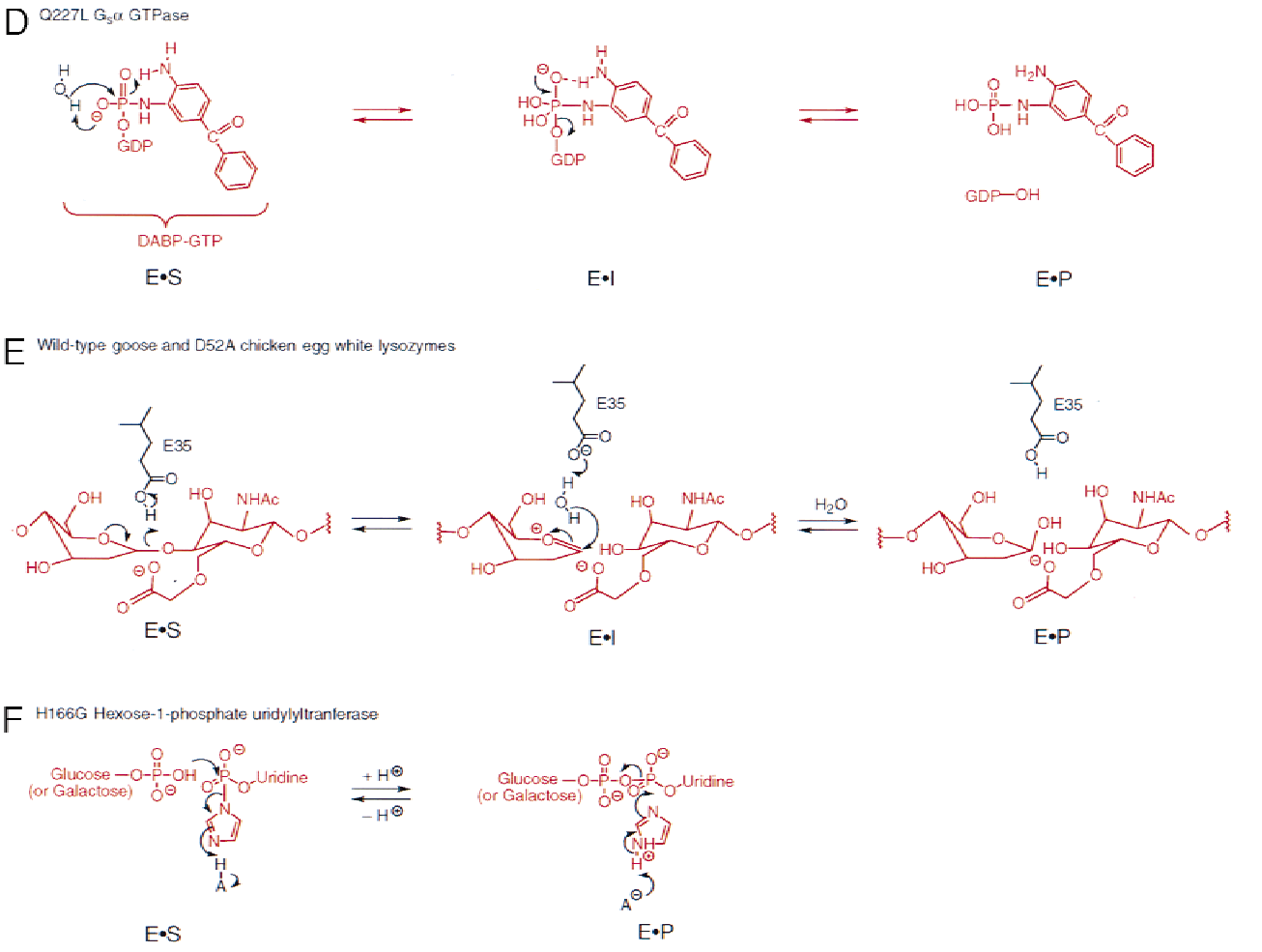


**Fig. 1.** *Continued.*

**Table 1.** *Examples of substrate-assisted catalysis by natural and engineered enzymes*

| Enzyme | Variant[a] | Substrate group | | Evidence for substrate-assisted catalysis[b] | References |
|---|---|---|---|---|---|
| | | Identity | Proposed role | | |
| **Serine proteases** | | | | | |
| Subtilisin BPN′ | H64A | P2 His, P1′ His | General base | 1, 2, 3, 4 | Carter & Wells (1987); Carter et al. (1989, 1991); Matthews & Wells (1993) |
| Trypsin | H57A | P2 His, P1′ His | General base | 1, 2 | Corey et al. (1995) |
| Elastase | H57A | P2 His | General base | 1, 2 | Dall'Acqua et al. (1999) |
| **Type II restriction endonucleases[c]** | | | | | |
| *Eco*RI | WT | Phosphate 3′ to scissile bond | General base | 1, 2, 5 | Jeltsch et al. (1992, 1993, 1995); Horton et al. (1998); Koziolkiewicz et al. (1992) |
| *Eco*RV | WT | Phosphate 3′ to scissile bond | General base | 1, 2, 5 | Jeltsch et al. (1992, 1993, 1995); Horton et al. (1998); Thorogood et al. (1996) |
| **GTPases** | | | | | |
| Ras p21 | WT | $\alpha$-phosphate | Stabilization of intermediate by hydrogen bond | 1, 6 | Schweins et al. (1994, 1995) |
| $G_s\alpha$ | Q227L | Amino | Stabilization of intermediate by hydrogen bond | 1, 2 | Zor et al. (1997, 1998) |
| **Lysozymes and chitinases** | | | | | |
| Chicken egg white lysozyme | D52A | Carboxyl | Electrostatic stabilization of oxocarbenium intermediate | 1, 2, 4 | Matsumura and Kirsch (1996) |
| Goose egg white lysozyme | WT | Carboxyl | Electrostatic stabilization of oxocarbenium intermediate | 1, 2 | Matsumura and Kirsch (1996) |
| Hevamine | WT | Carboxyl | Electrostatic stabilization of oxocarbenium intermediate | 1, 5 | Terwisscha van Scheltinga et al. (1995) |
| **Aminoacyl tRNA synthetases** | | | | | |
| Glutaminyl-tRNA synthetase | WT | Phosphate | General base | 1 | Perona et al. (1993) |
| Aspartyl tRNA synthetase | WT | Phosphate | General base | 1 | Cavarelli et al. (1994) |
| **Other enzymes** | | | | | |
| Hexose-1-phosphate uridylyltransferase | H166G | Imidazoyl | Leaving group | 1, 2 | Kim et al. (1990) |
| Serine carboxypeptidase WII | WT | Carboxyl | | 1 | Bullock et al. (1994) |
| Nitric oxide synthase | WT | Amino guanidine | Stabilization of oxo-iron intermediate by hydrogen bond | 1 | Crane et al. (1997) |
| Aspartate carbamoyltransferase | WT | Phosphate | General base | 1 | Gouaux et al. (1987); Gouaux and Lipscomb (1988) |
| Self-splicing intron | WT | Phosphate | Holds and orients water | 1 | Setlik et al. (1993) |
| RNase HI | WT | Phosphate | General base | 1 | Katayanagi et al. (1993) |
| Cytochrome P450eryF | WT | | Holds and orients water | 1 | Cupp-Vickery et al. (1996) |

[a] Wild-type.
[b] Supporting evidence for SAC: 1, structure-based prediction; 2, substrate analogs/specificity; 3, pH dependence of catalysis; 4, mutational analysis; 5, stereochemistry of reaction; 6, linear free energy relationships.
[c] Many type II restriction endonucleases likely undergo SAC (Jeltsch et al., 1995).

**Table 2.** *Efficient substrate-assisted catalysis by subtilisin by cooptimization of enzyme and substrate*[a]

| Residue position[b] | | | | | | | | $k_{cat}/K_m$ ($s^{-1}$ $M^{-1}$)[c] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 24 | 31 | 33 | 64 | 156 | 166 | 169 | 217 | sFAHF*p*na | sFAHY*p*na | sAAHY*p*na |
| C | Ile | Ser | A | Glu | Gly | Gly | Tyr | $6.2 \times 10^1$ | $4.8 \times 10^2$ | $2.1 \times 10^3$ |
| C | Ile | Ser | A | Glu | A | Gly | Tyr | $2.6 \times 10^2$ | $4.2 \times 10^1$ | ND |
| C | Ile | Ser | A | S | Gly | A | L | $2.2 \times 10^2$ | $1.0 \times 10^3$ | $4.0 \times 10^3$ |
| C | Ile | Ser | A | S | A | A | L | $6.7 \times 10^2$ | $2.9 \times 10^1$ | ND |
| C | L | Ser | A | S | Gly | A | L | $2.7 \times 10^2$ | ND[d] | $4.9 \times 10^3$ |
| C | Ile | T | A | S | Gly | A | L | $5.0 \times 10^2$ | ND | $4.8 \times 10^3$ |
| C | L | T | A | S | Gly | A | L | $9.0 \times 10^2$ | ND | $9.6 \times 10^3$ |
| C | Ile | Ser | His | Glu | Gly | Gly | Tyr | $3.6 \times 10^5$ | $9.7 \times 10^5$ | $7.3 \times 10^6$ |

[a]Data are taken from Carter et al. (1989, 1991) and P. Carter (unpubl. data).
[b]Wild-type and mutant residues are shown in three and one letter codes, respectively.
[c]Assays were performed at (25.0 ± 0.2) °C in the presence of 100 mM Tris-HCl (pH 8.6) and 4% (v/v) DMSO. The standard errors were ≤±15%.
[d]ND, not determined.

libraries based upon catalysis (Pedersen et al., 1998; Demartis et al., 1999). Such technologies may also allow optimization of various enzymes for efficient SAC.

Enhanced activity variants of H64A subtilisin have proved useful for site-specific proteolysis of fusion proteins (Carter et al., 1989; Carter, 1990; Forsberg et al., 1991, 1992) including in phage display (Matthews & Wells, 1993; Ward et al., 1996; Demartis et al., 1999). In addition, H64A subtilisin has recruited for autocatalytic zymogen activation by engineering its own pro sequence to contain a histidine residue (Shinde & Inouye, 1995). The practical feasibility of using H64A variants for site-specific proteolysis on a preparative scale has been substantially enhanced by their immobilization, thereby permitting recycling of the enzyme (Beck et al., 1994). In addition, an enhanced activity variant of H64A subtilisin, "Genenase" (Carter et al., 1989), has been purified in multigram quantities following high cell density culturing of *Bacillus amyloliquefaciens* in a fermentor (G. Weiss & P. Carter, unpubl. data).

*Trypsin and elastase: SAC with substrate histidines*

There are at least two distinct families of serine proteases, the "subtilisin-like" and the "trypsin-like" that arose from separate genes. The two families have distinctly different folds, but nevertheless possess catalytic triads that are virtually superimposable (Corey & Craik, 1992). This led to the proposal that SAC with a P2 histidine might also be applicable to trypsin (Carter & Wells, 1987). Corey et al. (1995) subsequently demonstrated SAC by trypsin with a P2 and also a P1′ histidine. H57A trypsin cleaves related peptides with P2 and P1′ histidines with very similar catalytic efficiencies, whereas activity was increased more than fourfold when a histidine was included at both P2 and P1′ positions (Corey et al., 1995).

An additional example of SAC is provided by human neutrophil elastase, another member of the trypsin family (Dall'Acqua et al., 1999). Histidine-containing substrates for H57A elastase (chymotrypsin numbering) were identified using a substrate phage library containing randomized sequences. Ten different phage-derived and related linkers were cleaved by H57A elastase, all with a P2 histidine. A fusion protein containing the phage-derived sequence, REHVVY, was cleaved by H57A elastase with a catalytic efficiency that is within 160-fold of that for the wild-type enzyme.

Aside from their exquisite preference for histidine, variants of subtilisin, trypsin, and elastase lacking the catalytic histidine share similar subsite specificity to their parent enzymes. For example, H64A, like wild-type subtilisin, has a strong preference for tyrosine and phenylalanine at P1 and also favors phenylalanine and alanine at P4. In the case of H57A trypsin, cleavage occurs with a histidine at either P2 or P1′ positions and either lysine or arginine at P1: HR↓, HK↓ and K↓H, R↓H (Corey et al., 1995). Finally, for H57A elastase, a strong preference was observed for threonine or valine at P1, as for the wild-type enzyme (Dall'Acqua et al., 1999).

It seems likely that SAC is broadly applicable to serine proteases in light of the three successful examples and the conservation of the catalytic triad geometry. Thus, it should be possible to generate a family of highly specific histidine-dependent proteases. Such "restriction endoproteases" have broad potential research and biotechnological applications including for peptide mapping, site-specific cleavage of fusion proteins, and perhaps activation of peptidyl prodrugs for cancer therapy (Chakravarty et al., 1983).

*Evolution of serine proteases*

It is exceedingly improbable that the catalytic triad of serine proteases evolved in a single step but likely involved functional intermediates that may have included SAC where a serine and aspartate were provided by the enzyme and the histidine by the substrate. SAC with a catalytic dyad comprising enzyme serine and substrate histidine is also possible, albeit with slightly reduced catalytic efficiency compared to SAC with the catalytic aspartate present (Table 3). Surprisingly, the catalytic efficiency of a catalytic dyad in subtilisin, with activated *p*-nitroanilide substrates at least, is very similar whether enzyme or substrate contributes the histidine.

*Type II restriction endonucleases*

SAC is a more widespread and indeed more useful phenomenon than has perhaps previously been appreciated, as evidenced by naturally occurring enzymes. For example, many type II restriction endonucleases—a mainstay of molecular biologists—are believed to utilize SAC. Type II enzymes are homodimers that recognize a 4–8 base pair palindromic sequence in double-stranded DNA and,

**Table 3.** *Possible functional intermediates in the evolution of the catalytic triad of serine proteases*[a]

| Subtilisin variant | Catalytic triad[b] S | H | D | $k_{cat}/K_M$ ($s^{-1}$ $M^{-1}$) sFAHF-*p*na | sFAQF-*p*na |
|---|---|---|---|---|---|
| WT | + | + | + | $2.6 \times 10^5$ | $1.7 \times 10^5$ |
| S24C | + | + | + | $3.6 \times 10^5$ | $1.5 \times 10^5$ |
| S24C:H64A | + | − | + | $6.2 \times 10^1$ | $2.7 \times 10^{-1}$ |
| S24C:H64A:D32A | + | − | − | 9 | 6.2 |
| S24C:D32A | + | + | − | $1.1 \times 10^1$ | 7.9 |

[a] Assays were performed at (25.0 ± 0.2) °C in the presence of 100 mM Tris-HCl (pH 8.6) and 4% (v/v) DMSO (Carter et al., 1991). The standard errors were ≤±15%.

[b] +, Wild-type residue; −, alanine.

in the presence of $Mg^{2+}$ ions, cleave both strands within or adjacent to the recognition site to yield 3′ hydroxyl and 5′ phosphate ends (Pingoud & Jeltsch, 1997). Cleavage of double-stranded DNA by type II restriction endonucleases yields either 3′ or 5′ overhangs or no overhang ("blunt ends"), depending upon the relative position of cleavage within the two strands. Approximately 3,000 type II restriction endonucleases have been identified with 221 different cleavage specificities (Roberts & Macelis, 1999).

### *Catalytic mechanism of EcoRV and EcoRI*

The mechanism of a few type II restriction endonucleases have been studied although none is known in precise detail. Nevertheless, it is widely accepted that a general base activates a water molecule to create a hydroxide ion that engages in nucleophilic attack of specific phosphorus atom of the DNA substrate (Pingoud & Jeltsch, 1997). Phosphodiester bond hydrolysis by *Eco*RI (Connolly et al., 1984) and *Eco*RV (Grasby & Connolly, 1992) occurs with stereochemical inversion at the phosphorus atom. This is consistent with a pentacoordinate phosphorus transition state in which attack of a water molecule occurs in-line with the 3′-leaving group. For DNA complexes with both *Eco*RI and *Eco*RV, a modeled in-line water molecule can form a hydrogen bond to the pro-$R_p$ oxygen atom of the phosphate group that is immediately 3′ to the scissile bond. These structural studies led to the hypothesis that DNA cleavage by *Eco*RI and *Eco*RV involves SAC with the proximal substrate phosphate (Jeltsch et al., 1992, 1993).

Evidence for a catalytic role for a substrate phosphate in type II restriction endonucleases is provided by cleavage experiments using modified substrates lacking a negatively charged oxygen on this phosphate or missing this group entirely. For example, synthetic *Eco*RI and *Eco*RV substrates lacking this 3′ proximal phosphate in one strand bind efficiently to corresponding enzymes, but are cleaved only in the unmodified strand. Similarly, substrates with a hydrogen phosphonate replacement at the 3′ proximal phosphate are cleaved more than $10^4$-fold more slowly than the corresponding unmodified substrates (Jeltsch et al., 1993). Involvement of a substrate phosphate has been suggested for 11 of 17 type II enzymes evaluated with hemi-modified methylphosphonate substrates (Jeltsch et al., 1995).

Although the 3′ proximal substrate phosphate is widely believed to participate in SAC by several type II restriction endonucleases, its precise role is still debated. The substrate phosphate was initially proposed to both position and deprotonate an attacking water molecule (Jeltsch et al., 1992) (Fig. 1B). The negative charge of the transition state was predicted to be stabilized by a semi-conserved lysine residue and a $Mg^{2+}$ ion, the later being coordinated by two conserved acidic residues. The 3′ leaving group was suggested to be protonated by a water molecule activated by a second catalytic $Mg^{2+}$ ion. Support for the notion that a substrate phosphate deprotonates water is provided by phosphorothioate substrates. Cleavage was observed for a charged sulfur atom capable of deprotonating although not precisely positioning a water molecule. In contrast, cleavage was not observed with an uncharged phosphoryl oxygen capable of positioning but not deprotonating water (Koziolkiewicz & Stec, 1992; Thorogood et al., 1996).

A weakness of the original SAC model for type II restriction endonucleases (Fig. 1B) is that the acidity of the substrate phosphate ($pK_a = 2$) makes it poorly suited to its proposed role of deprotonating a water molecule at neutral pH. This difficulty might be overcome if the protein environment significantly perturbs the $pK_a$ of the substrate phosphate. An alternative SAC model is that the substrate phosphate coordinates a water molecule for activation by a second catalytic $Mg^{2+}$ ion (Horton et al., 1998). However, this second model is unsatisfactory since others have concluded that only a single metal ion is involved in catalysis (Groll et al., 1997; Stahl et al., 1998).

In addition to restriction endonucleases, SAC involving a substrate phosphate as a general base has been proposed for several other enzymes including G proteins (Schweins et al., 1994, 1995), aminoacyl tRNA synthetases (Perona et al., 1993; Cavarelli et al., 1994), RNase HI (Katayanagi et al., 1993), aspartate carbamoyltransferase (Gouaux et al., 1987; Gouaux & Lipscomb, 1988), and self-cleaving group I intron ribozymes (Setlik et al., 1993). G proteins are the best substantiated of these additional candidate enzymes utilizing a substrate phosphate for SAC (see below).

### *GTPases*

Guanidine nucleotide-binding proteins (G proteins) are crucial to numerous signal transduction pathways involving GTP hydrolysis cycles. The superfamily of G proteins includes small *Ras*-like GTPases (Bos, 1997) as well as heterotrimeric GTPases comprising $\alpha$-, $\beta$-, and $\gamma$-subunits (Wilkie & Yokoyama, 1994; Neer, 1995; Wall et al., 1995; Lambright et al., 1996). A conserved active site glutamine residue plays a prominent, although imprecisely defined role, in the activity of these GTPases (Gibbs et al., 1988; Graziano & Gilman, 1989; Masters et al., 1989; Pai et al., 1990). Structural studies have implicated this glutamine residue in the formation and stabilization of a transition state involving a pentavalent $\gamma$-phosphate (Prive et al., 1992; Coleman et al., 1994; Sondek et al., 1994).

#### *Ras*

Ras p21 is a G protein that plays a major role in the regulation of cell growth and differentiation. Activation of receptors coupled to *Ras* p21 and the subsequent GTP hydrolysis-regeneration cycles elicit conformational changes in *Ras* that allow (GTP-bound state) or forbid (GDP-bound form) the specific binding of effector proteins (Vojtek et al., 1993; Bos, 1997). *Ras* p21 will slowly convert bound GTP to GDP ($k_{cat} = 5 \times 10^{-4}$ $s^{-1}$) (Temeles et al., 1985). This hydrolysis is accelerated more than $10^5$-fold upon binding to GTPase-activating protein, thereby switching off the growth signal (Gideon et al., 1992). *Ras* mutations at positions 12, 13, and 61 are

commonly associated with human cancers, including 90% of pancreatic adenocarcinomas and 50% colorectal carcinomas (Bos, 1997). Such mutations greatly reduce the catalytic efficiency of the *Ras* GTPase and prevent the growth signal from being switched off (Der et al., 1986; Landis et al., 1989; Lowy & Willumsen, 1993).

Hydrolysis of GTP by Ras requires the nucleophilic attack of a water molecule (Pai et al., 1990). Much effort has been devoted to the identification of the general base that activates the attacking water molecule. Neither theoretical (Langen et al., 1992) nor experimental (Chung et al., 1993; Schweins et al., 1995) studies on *Ras* has revealed a plausible candidate residue in this enzyme that might serve as an activating base. Using energy simulation models, it was suggested that GTP itself activates a water molecule through an oxygen atom attached to its $\gamma$-phosphate (Schweins et al., 1994). The strong correlation between the reaction rate of the Ras p21 G protein and the proton abstraction potential of the protein-bound GTP (Schweins et al., 1995) support this hypothesis. In this model, a conserved active site glutamine residue stabilizes the transition state of the reaction. Thus, GTP hydrolysis by *Ras* apparently involves SAC. This SAC hypothesis is applicable to distantly-related G proteins, such as EF-tu and transducin $\alpha$, which contain ordered water molecules in close proximity to an oxygen of the $\gamma$-phosphate in analogous positions to that found in the structure of *Ras* itself (Kjeldgaard et al., 1993; Noel et al., 1993).

*$G_s\alpha$*

Heterotrimeric G proteins act as molecular switches in that hydrolysis of bound GTP converts them from an active dissociated to inactive associated states (Gilman, 1987; Bourne et al., 1991; Neer, 1995):

$$\underset{\text{active}}{\text{GTP-}\alpha + \beta\gamma} \Leftrightarrow \underset{\text{inactive}}{\text{GDP-}\alpha\beta\gamma}.$$

Activation of a G protein-coupled receptor leads to exchange of GDP for GTP and elicits conformational changes leading to the dissociation of the GTP-bound state of the G$\alpha$ subunit from the $\beta\gamma$ complex (Wall et al., 1995). Hydrolysis of the G$\alpha$-bound GTP, through G$\alpha$ intrinsic GTPase activity or GAP (Dohlman & Thorners, 1997), restores the inactive GDP-$\alpha\beta\gamma$ complex.

SAC has been demonstrated in the context of heterotrimeric G proteins when an inactive mutant of the protein $G_s\alpha$ (Q227L) was shown to be rescued by an amino group-containing GTP analog (DABP-GTP) fulfilling the role of the missing glutamine (Zor et al., 1997). A more detailed dissection of the structural requirements of such analogs followed (Zor et al., 1998), reinforcing the role of the glutamine residue in transition state stabilization. The proposed SAC model for Q227L $G_s\alpha$ with DABP-GTP closely parallels that with wild-type *Ras* (Fig. 1C,D). Q227L $G_s\alpha$ with DABP-GTP represents the most efficient example of SAC to date in that the activity of the mutant protein was fully restored to that of the parent wild-type enzyme. The application of SAC to engineer the activity of G proteins has therapeutic implications. Indeed, it raises the possibility of restoring activity to mutated forms of G proteins, whose activity has been lost through mutation (Der et al., 1986).

*Lysozymes*

Lysozymes hydrolyze glycosidic bonds in bacterial peptidoglycans and chitin substrates. Participation of lysozymes in SAC has only recently been proposed (Matsumura & Kirsch, 1996), despite extensive structure-function studies of these enzymes. The active site residue, D52, is very well conserved in chicken-type lysozymes. Substitution of D52 with asparagine (Malcolm et al., 1989) or alanine (Matsumura & Kirsch, 1996) in chicken egg white lysozyme reduces the catalytic activity by ~20-fold. Thus, residue D52 is important but not crucial to catalysis by chicken-type lysozymes. D52 appears to stabilize the oxocarbenium intermediate (Vernon, 1967; Rosenberg & Kirsch, 1981) and distort the saccharide ring into a conformation that resembles the oxocarbenium-ion transition state (Strynadka & James, 1991).

Goose-type, unlike chicken-type, lysozymes lack an aspartate at residue 52 and also exhibit a strong preference for peptide-substituted substrates (Arnheim et al., 1973), implicating the peptide portion of the substrates either directly or indirectly in catalysis. Goose and D52A chicken egg white lysozymes have modestly higher (approximately twofold) catalytic activities toward carboxymethylchitin than with ethylene glycol-chitin (Matsumura & Kirsch, 1996). This led to the proposal of a SAC model in which a carboxyl group on a substrate emulates D52 (Fig. 1E). Molecular modeling supports this hypothesis by demonstrating that a substrate carboxylate can be positioned in the active site of lysozyme at the position normally occupied by D52 (Matsumura & Kirsch, 1996).

Independently, SAC was postulated in hevamine, a plant enzyme with chitinase and lysozyme activity, based upon the structure of this enzyme complexed with the transition state analog/inhibitor allosamidin (Terwisscha van Sheltinga et al., 1995). A SAC model was posited in which an oxocarbenium ion intermediate is stabilized by a partial negative charge induced on the carbonyl oxygen of the *N*-acetyl group of the chitin substrate. No enzyme residue could be identified that might potentially fulfill this role. Alternatively, the reaction intermediate might be stabilized by the formation of a covalent bond between the carbonyl oxygen and the C1 atom of the substrate.

*Hexose-1-phosphate uridylyltransferase*

The enzyme, hexose-1-phosphate uridylyltransferase catalyzes the interconversion of UDP-galactose and glucose-1-phosphate with UDP-glucose and galactose-1-phosphate via an intermediate in which UMP forms a covalent adduct with H166. Removal of this catalytic histidine, as in the H166G variant, abolishes activity against these substrates. However, the H166G mutant, unlike the wild-type enzyme, can utilize uridine-5′-phosphoimidazolate as a substrate together with glucose-1-phosphate or galactose-1-phosphate to form UDP-glucose and UDP-galactose, respectively (Kim J et al., 1990) (Fig. 1F). Thus, the H166G variant employs SAC to catalyze a new reaction by a different catalytic mechanism from that of its parent enzyme.

## Conclusions and future perspectives

SAC is more prevalent than previously appreciated, particularly with naturally occurring enzymes. SAC has been most convincingly demonstrated with hydrolytic enzymes, but has also been postulated for many other types of enzyme including aminoacyl-tRNA synthetase tRNA (Perona et al., 1993; Cavarelli et al., 1994), cytochrome P450eryF (Cupp-Vickery et al., 1996), and nitric oxide synthase (Crane et al., 1997). SAC can apparently utilize several different mechanisms including general base catalysis and stabil-

ization of intermediates (Table 1). Substrates can also assist in catalysis by orienting water molecules.

SAC with engineered enzymes provides a way of drastically changing their substrate specificity (Carter & Wells, 1987) and in some cases modifying the reaction catalyzed (Kim J et al., 1990). In one case at least, the catalytic efficiency of SAC is comparable to that of the wild-type enzyme (Zor et al., 1997). In other cases, it may be possible to increase the efficiency of SAC by exploiting the recent rapid progress in molecular evolution technologies (Patten et al., 1997).

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