(found); melting point (*RS*) = 101° to 102°C, for (*SS*) = 112° to 114°C.

12. Phenanthrene was treated with an equimolar amount of osmium tetroxide and two equivalents of pyridine in benzene to yield the osmate ester after 7 days of stirring at 25°C. The ester was hydrolyzed with a solution of mannitol and potassium hydroxide in methylene chloride/water [R. Criegee, B. Marchand, H. Wannowius, *Ann. Chem.* **550**, 99 (1942)], and the resulting *cis*-diol was isolated by silica-gel chromatography (1:1, hexanes:ethyl acetate) after removing excess pyridine by coevaporation with *n*-heptane. Adipic acid monomethyl ester was converted to the acid chloride by treatment with an equimolar amount of oxalyl chloride in methylene chloride with a catalytic amount of dimethylformamide. The corresponding aldehyde was obtained by hydrogenation in the presence of one equivalent of 2,6-lutidine with 10% palladium on carbon in tetrahydrofuran and purified by silica-gel chromatography (3:1, hexanes:ethyl acetate). The aldehyde was combined with one equivalent of the *cis*-diol and a catalytic amount of *p*-toluenesulfonic acid monohydrate in tetrahydrofuran and stirred overnight at 25°C in the dark. The resulting acetal was purified by silica-gel chromatography (1:1, hexanes:ethyl acetate) and hydrolyzed with lithium hydroxide to yield the lithium salt of acid **3**. The ligand was dissolved in 0.1 M citric acid and extracted into chloroform to generate the free acid before coupling to the column matrix.
13. Analog **3** was coupled to both Affi-Gel 102 (Bio-Rad) and EAH Sepharose 4B (Pharmacia). The agarose matrices were slowly equilibrated in acetone, and then, for each milliliter of solvated matrix, 2.5 μmol of **3**, and equimolar amounts of benzotriazolyl-*N*-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP), *N*-hydroxybenzotriazole (HOBT), and diisopropylethylamine were added. The mixture was stirred for 7 hours at 20°C with a Techne MCS-104L biological stirrer, after which time 300 μmol per milliliter of matrix of both triethylamine and acetic anhydride were added to block unreacted amines. The beads were washed five times with five bed volumes of acetone and stored at −20°C. Control columns were blocked with acetic anhydride and triethylamine in a similar fashion. In order to determine the extent of derivatization of the support, we incubated monoclonal antibodies specific for analog **3** with the affinity supports in phosphate-buffered saline (PBS) containing 10% calf serum for 1 min. The beads then were washed four times with five volumes of PBS and analyzed with alkaline phosphatase–conjugated goat antibodies to mouse immunoglobulin.
14. The cDNAs were prepared from eluted RNAs by reverse transcription. The reactions were carried out in 20 μl of buffer [10 mM tris-HCl (pH 8.9), 50 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin, and 0.1% Triton X-100 containing 1.25 mM of each deoxynucleotide triphosphate, 5 μM of each 5′ and 3′ extension primer, and 10 U of avian reverse transcriptase] for 20 min at 50°C. The PCR reactions were carried out in the identical tube after the addition of 180 μl of the above reaction buffer with the addition of 5 U of Taq polymerase. Reactions were cycled through 1 min at 94°C and 1 min at 54°C until ~10 μg of double-stranded DNA was obtained. The PCR reaction mixture (50 μl) was added directly to 250 μl of a transcription cocktail containing 40 mM tris-HCl (pH 7.8), 5 mM dithiothreitol, 20 mM $MgCl_2$, 5 mM of each ribonucleoside triphosphate, and 3750 U of T7 RNA polymerase and then incubated for 2 hours at 41°C to yield ~1 mg/ml of full-length transcript.
15. The seventh round of selection was carried out with the TS analog **3** immobilized on an EAH Sepharose 4B support (which contains an extended tether and non–cross-linked agarose). After this final round, less than 1% of the enriched library bound the acetylated Sepharose control column, whereas >85% bound the affinity support.
16. The plasmid pIK-1A4 is a pUC19 derivative containing a Sac I–Bam H1 cloning site after the class three T7 RNA polymerase promoter.
17. Reactions were followed by HPLC with a Microsorb C18 reversed-phase column (Rainin Instruments) and a mobile phase of 70% acetonitrile in water over 11 min by monitoring ultraviolet absorbance at 220 nm. Product **2** was identified by co-injection with authentic material.
18. I. H. Segel, *Enzyme Kinetics* (Wiley, New York, 1975), p. 46.
19. Inhibition reactions were carried out at 50, 100, 150, 200, 250, 300, 350, 400, 450, and 500 μM substrate **1** and 10 μM RNA in assay buffer at 28°C.
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22. There are a maximum of 10^{15} or roughly 4^{25} sequences in the RNA library. If 25 bases are strictly required for catalysis, one might expect to find a single unique sequence in the library.
23. Selection of the RNA library against TS analog **3** at pH 7.5 under otherwise identical conditions afforded 2 out of 12 clones with the identical sequence to clone AA6.
24. We are grateful for financial support for this work from the Office of Naval Research (grant #N00014-91-J-1130) and from the Director, Office of Health Effects Research, of the U.S. Department of Energy under contract no. DE-AC03-76SF00098. J.R.P. was supported by NIH Biotechnology Training Grant GM08352A. We thank J. Szostak and J. Lorsch for helpful discussions.

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A Low-Barrier Hydrogen Bond in the Catalytic Triad of Serine Proteases

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Spectroscopic properties of chymotrypsin and model compounds indicate that a low-barrier hydrogen bond participates in the mechanism of serine protease action. A low-barrier hydrogen bond between Nδ1 of His^{57} and the β-carboxyl group of Asp^{102} in chymotrypsin can facilitate the formation of the tetrahedral adduct, and the nuclear magnetic resonance properties of this proton indicate that it is a low-barrier hydrogen bond. These conclusions are supported by the chemical shift of this proton, the deuterium isotope effect on the chemical shift, and the properties of hydrogen-bonded model compounds in organic solvents, including the hydrogen bond in *cis*-urocanic acid, in which the imidazole ring is internally hydrogen-bonded to the carboxyl group.

The mechanism by which the catalytic triad in serine proteases catalyzes the hydrolysis of peptide bonds has long been debated. In chymotrypsin, the triad consists of Ser^{195}, His^{57}, and Asp^{102}. Spectroscopic evidence indicates that a strong hydrogen bond (a low-barrier hydrogen bond or LBHB) links His^{57} and Asp^{102} in the protonated state of the catalytic triad. The formation of an LBHB between His^{57} and Asp^{102} in the course of catalysis should increase the reactivity of His^{57} as a general base. We postulate a new mechanism in which the formation of this LBHB facilitates nucleophilic attack by the β-OH group of Ser^{195} on the acyl carbonyl group of substrates.

Hydrogen bonds have been classified in three types (*1*): (i) weak or conventional (2.4 to 12 kcal mol^{-1}); (ii) strong or low-barrier (12 to 24 kcal mol^{-1}); and (iii) very strong or single-well (>24 kcal mol^{-1}). The potential energy wells in Fig. 1 represent these types for a system where a proton between two heteroatoms is exchanged $\mathbf{A-H\cdots B} \leftrightarrows \mathbf{A\cdots H-B}$. An example of very strong hydrogen bonding is provided by hydrogen difluoride ($[FHF]^-$) (*1*, *2*), in which the distance between fluorine atoms is 2.26 Å and the hydrogen bond energy is 37 kcal mol^{-1}. Low-barrier and single-well hydrogen bonds may be represented as $\mathbf{A|||H|||B}$.

Strong hydrogen bonds can form when the distance between the heteroatoms ($R_{A\cdots B}$) is less than the sum of the van der Waals radii (<2.55 Å for O–H–O and <2.65 Å for O–H–N) and the change in the negative logarithm of the acid constant (ΔpK_a) between the heteroatoms is near 0 (*1*, *3*, *4*). Medium effects are important because values for pK_a are dependent on solvent; that is, variant pK_a's of two acidic groups in water may become similar in an organic solvent or in a crystalline state.

The most definitive physical characterization of an LBHB is the measurement of $R_{A\cdots B}$ by x-ray crystallography or neutron diffraction. In most cases, however, the structures of proteins are not known with enough accuracy to distinguish values of $R_{A\cdots B}$ between 2.5 Å and 2.7 Å. Of the four other physicochemical parameters for characterizing LBHBs (*1*), the most unambiguous is the nuclear magnetic resonance (NMR) chemical shift δ_H for a participating proton, which ranges from 16 to 20 parts per million (ppm). Three parameters depend on the effects of deuterium on the hydrogen bond. The isotope effect on infrared stretching frequencies is the ratio ν_{AH}/ν_{AD}, the isotope effect on the NMR chem-

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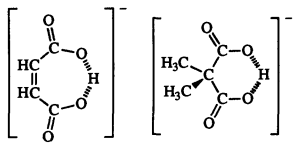
ical shift is $\Delta[\delta_H - \delta_D]$, and the fractionation factor for the exchange reaction A–H + $D_2O \rightleftharpoons$ A–D + HOD is ϕ. LBHBs often exhibit values for these isotope effects that deviate from the norm for weak hydrogen bonds.

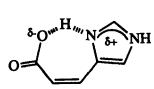
The NMR chemical shift characterizes LBHBs in simple compounds. The acidic proton in the tetrabutylammonium salt of hydrogen maleate (**1**) exhibits an NMR chemical shift of more than 20 ppm in several organic solvents (*5*, *6*).



1 **2**

We measured the chemical shifts of the acidic protons in various salts of hydrogen maleate (**1**) and hydrogen 2,2-dimethylmalonate (**2**) (Table 1), which show that the chemical shifts depend on the nature of the counter cation and, to a lesser extent, the solvent. The tetrabutylammonium salts of both compounds exhibit chemical shifts of 19.5 to 20.3 ppm in four organic solvents. For the tetrabutylammonium salts in aprotic solvents, the only possible hydrogen bonds are the intramolecular bonds in **1** and **2** or intermolecular bonds in bimolecular complexes. Complexation would be dependent on concentration, but the values of δ_H for these compounds do not depend on concentration (Table 1). The hydrogen bonding imidazolium counterion introduces other possibilities for hydrogen bonding in hydrogen maleate (**1**) and leads to values for δ_H as small as 12.2 ppm. Our data (Table 1) indicate that intramolecular LBHBs are formed when the value for ΔpK_a is near 0, geometric factors are favorable, and alternative hydrogen bonding possibilities are not available. These conditions may be met within the microenvironment of an enzymatic active site.

Examples of intramolecular LBHBs of the type **N**:::**H**:::**O** in simple compounds have been described (*1*). We discovered a new one, *cis*-urocanic acid (*7*), the geometrical isomer of the naturally occurring *trans*-urocanic acid.



cis-Urocanic acid

The acidic proton in *cis*-urocanic acid resonates at 17.4 ppm in dimethyl sulfoxide (DMSO)–d_6, 17.2 ppm in acetonitrile-d_3, and 16.9 ppm in acetone-d_6. The trans isomer does not exhibit a low-field signal in organic solvents. The LBHB in *cis*-urocanic acid may serve as a first approximation to a model for the His-Asp component of the triad in serine proteases.

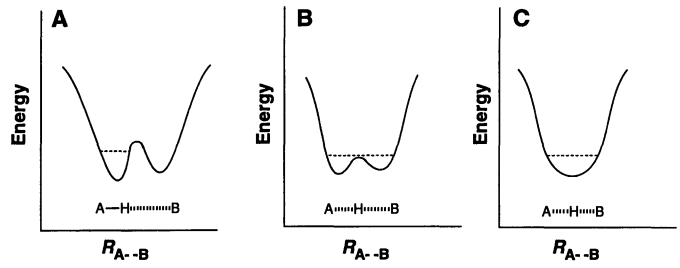


Fig. 1. Diagrams of the hydrogen-bonded heteroatoms as a function of $R_{A\cdot\cdot B}$. (**A**) The heteroatoms are separated by a distance corresponding to a weak hydrogen bond. The energy well separating atom A from hydrogen is deep and narrow, so that hydrogen is bonded covalently with atom A. (**B**) The heteroatoms are close together so that the energy barrier between them is low. The energy for binding the hydrogen atom places it at or slightly above the barrier between the heteroatoms in a strong LBHB. (**C**) The heteroatoms are so close together that there is no energy barrier and the hydrogen atom is equally bonded to each heteroatom in a very strong, no-barrier hydrogen bond.

The unusually low field proton signal in the NMR spectrum of chymotrypsin appears well downfield from all other protons in the enzyme, exhibiting a chemical shift (δ_H) of 15 ppm at pH 9 and 18 ppm at pH 4 (*8*). This signal was assigned to the proton engaged in hydrogen bonding between His^{57} and Asp^{102}. Protons bonded to nitrogen or oxygen typically exhibit chemical shifts of 9 to 12 ppm; that in imidazole dissolved in $CDCl_3$ is 11.4 ppm (*9*) and that in the imidazolium ion is 12.2 ppm (Table 1). On the basis of its chemical shift, the proton linking His^{57} and Asp^{102} must be very deshielded, although it has not heretofore been interpreted in terms of an LBHB.

The low-field signal in chymotrypsin was confirmed and extended to trypsin, where the chemical shift was 18 ppm at pH 3 (*10*). The assignment of this signal to the proton linking histidine and aspartate in the catalytic triad was proved by its coupling to $^{15}N\delta1$ in α-lytic protease (*11*). In basic solutions, the imidazole ring of His^{57} is uncharged, and in acidic solutions it is protonated and positively charged. It is reasonable to assign the signal at 18 ppm observed at pH 3 to protonated His^{57} and that at 15 ppm observed at pH 9 to unprotonated His^{57}.

We confirmed the chemical shift of 18.3 ppm for α-chymotrypsin at pH 3.5 and 5°C. Moreover, we also observed a signal δ_D at 17.3 ppm for the deuteron linking Asp^{102} and His^{57} in D_2O (pD 3.5 and 5°C; δ_D referenced to acetone-d_6). The isotope effect on the chemical shift, $\Delta[\delta_H - \delta_D] = +1.0 \pm 0.4$ ppm, is within the range of 0.5 to 0.9 ppm observed for LBHBs in simple molecules (*1*). An isotope effect near 0 would have been indicative of a weak hydrogen bond or of a very strong, single-well hydrogen bond. The chemical shift and isotope effect for this proton mean that His^{57} and Asp^{102} are linked by an LBHB. We were interested in the strength of this bond and what it means to the chemical reactivity of the catalytic triad.

The stabilization energies for LBHBs and single-well hydrogen bonds are large, ranging up to values for weak covalent bonds. A gas-phase hydrogen bond energy

Table 1. Proton NMR data for intramolecular hydrogen bonds in hydrogen maleate and hydrogen dimethylmalonate. THF-d_8, tetrahydrofuran; *n*-Bu, normal butyl.

Substrate	^{1}H NMR chemical shift [O–H–O] (ppm) dissolved in			
	$CDCl_3$	DMSO-d_6	CD_3CN	THF-d_8
	*Hydrogen maleate (**1**-X)*			
1-N(*n*-Bu)$_4^+$				
15 mM	20.3	20.2	20.3	20.2 (br)
0.5 mM	20.2	20.2	20.3	20.3 (br)
1-Na^+				
15 mM		20.1		
0.5 mM		20.2		
1-HN(*n*-Bu)$_3^+$				
15 mM		19.9	19.0	17.6 (br)
0.5 mM		19.9	19.3	17.2 (br)
1-imidazolium				
15 mM		14.8 (br)		
0.5 mM		12.2 (br)		
	*Hydrogen dimethylmalonate (**2**-X)*			
2-N(*n*-Bu)$_4^+$				
15 mM	19.5	19.6	19.6	19.4 (br)
0.5 mM	19.5	19.6	19.6	19.2 (br)

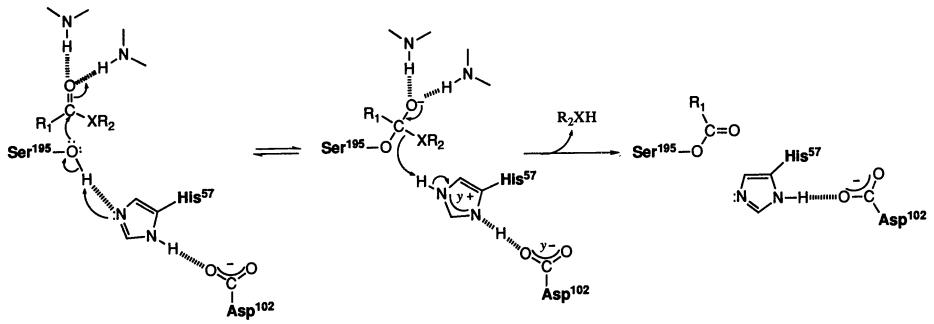
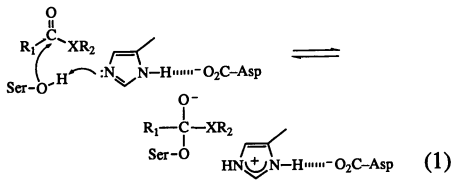


Fig. 2. The LBHB-facilitated general base mechanism for the acylation of chymotrypsin. In this mechanism, the formation of an LBHB between Asp^{102} and Nδ1 of His^{57} facilitates the action of Nϵ2 in abstracting the proton from the serine–β-OH group. The catalytic triad in the tetrahedral addition intermediate is stabilized by the LBHB, and this lowers the activation energy for its formation.

of 29.8 kcal mol^{-1} has been calculated for hydrogen maleate (1) (*12*). This is almost certainly a single-well hydrogen bond and is stronger than the LBHB in chymotrypsin, which may have a stabilization energy of 12 to 20 kcal mol^{-1}.

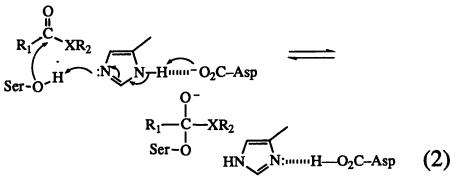
Consider how the formation of an LBHB between His^{57} and Asp^{102} can facilitate catalysis. The acylation of Ser^{195} by substrates is an essential process in peptide hydrolysis by chymotrypsin. Both specific substrates, such as *N*-acetyltryptophan amide, and nonspecific substrates, such as *p*-nitrophenylacetate, acylate Ser^{195}, and hence it is clear that the serine OH group is more reactive toward all substrates than the histidine imidazole ring. Aliphatic OH groups are less nucleophilic than imidazole rings, so that a basic question in understanding chymotrypsin is how the catalytic triad imparts high nucleophilic reactivity to the serine–β-OH group in the first step, the formation of the tetrahedral adduct between Ser^{195} and the substrate.

According to the conventional general base mechanism



(1)

the substrate carbonyl group is postulated to be close to the β-OH group of Ser^{195}, with His^{57} fixed in the right position to abstract the OH proton. The Asp^{102} residue is postulated to hold the imidazole ring of His^{57} in position through hydrogen bonding, so that the substrate is constrained to react with Ser^{195} and cannot acylate the more reactive His^{57}. The charge relay mechanism was first postulated by Blow *et al.* to explain the role of Asp^{102}, which they discovered in the crystal structure (*13*). In this mechanism, the role of Asp^{102} is expanded to include its action as a base in the abstraction of a proton from His^{57}. In the modified charge relay mechanism,

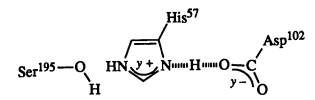


(2)

two concerted proton transfers are postulated to take place in the transition state for the formation of the tetrahedral addition intermediate.

Nonenzymatic chemical models of the catalytic triad failed to support the charge relay mechanism (*14, 15*). NMR data on the carbon-bonded protons of the catalytic histidine in chymotrypsin and trypsin supported the conventional general base mechanism because of the retention of positive charge on the imidazole ring (*16*). Neutron diffraction data further strengthened the case for histidine being the general base (*17*).

The low-field proton identified in the protonated catalytic triad by its chemical shift of 18.3 ppm offers a way to understand the mechanism by which high nucleophilic reactivity is conferred on Ser^{195}. The structure of the triad at pH 3.5 can be formulated as follows



with His^{57} linked to Asp^{102} by an LBHB. We designate the charges as $y+$ and $y-$, where $1 > y \geq 0.5$. The value of y will be 0.5 if the LBHB is a symmetrical, single-well bond. If it is slightly asymmetric, y will be larger than 0.5 but less than 1.0.

Because LBHBs are observed in model compounds only in the absence of water, the protonated triad of serine proteases must be essentially sequestered from the medium. Its microenvironment must be favorable for an LBHB in other respects as well. Consider the requirement that ΔpK_a must be near 0. The pK_a's for the carboxyl group (4.8) and for the imidazolium ion (7.0) differ in water. However, in nonaqueous and apolar microenvironments (such as an active site), the pK_a's for neutral acids such as carboxyl groups can be elevated and those for positively charged acids such as the imidazolium ion may be depressed (*18*). Thus, the pK_a's of Asp^{102} and His^{57} in the active site of chymotrypsin can be similar or identical, and protonation should then lead to the sharing of the proton in an LBHB linking His^{57} and Asp^{102}. The NMR properties of this proton indicate this to be the case.

Histidine-57 is protonated in the first step of the conventional general base mechanism for the acylation of chymotrypsin (Eq. 1), and it is reasonable to expect the imidazolium ring in this intermediate to engage in low-barrier hydrogen bonding with Asp^{102} for the same reasons that the LBHB is formed in the protonated triad. The chemical shifts for this proton in analogs of the tetrahedral complex support this assignment. For example, the values of δ_H for the low-field proton in complexes of chymotrypsin with benzeneboronic acid and 3-phenylethylboronic acid are 16.5 and 17.2 ppm, respectively (*19*). A very close analog of the tetrahedral intermediate is the addition complex of *N*-acetyl-L-Leu-L-Phe trifluoromethyl ketone with Ser^{195} of chymotrypsin, in which the value of δ_H for the low-field proton is 18.7 ppm (*20*). These chemical shifts rule out both mechanisms of Eqs. 1 and 2. In the conventional general base mechanism, the chemical shift for the imidazolium ion in the tetrahedral complex would be 12 ppm (Table 1). In the charge relay mechanism, the chemical shift for the imidazole ring in the intermediate would be 11 ppm (*9*). The observed value of more than 18 ppm corresponds to an LBHB.

We postulate the LBHB-facilitated general base mechanism for the acylation of Ser^{195} in chymotrypsin (Fig. 2). In this mechanism, Nϵ2 of histidine abstracts the proton from the β-OH group of serine in the transition state for its addition to the substrate-carbonyl group. In the tetrahedral intermediate, Nδ1 of histidine forms an LBHB with the β-COOH of Asp^{102}, and this stabilizes the imidazole-COOH portion of the triad. To the extent that the transition state is stabilized by the incipient LBHB, the activation energy is reduced and the rate is increased. Formation of the acyl enzyme in the next step requires the abstraction of a proton from the imidazole ring, so that the LBHB is disrupted in the acyl enzyme.

The catalysis provided by the LBHB linking His^{57} and Asp^{102} does not supersede other means of catalysis. The LBHB-facilitated mechanism (Fig. 2) includes weak hydrogen bonds between the tetrahedral oxyanion and peptide amide N–H bonds contributed by Gly^{193} and Ser^{195} (*21*), which stabilize the incipient tetrahedral adduct on the substrate side of the transi-

tion state (*22*). On the enzymatic side of the same transition state, the catalytic groups participating in proton abstraction are stabilized by the LBHB.

In the LBHB-facilitated mechanism (Fig. 2), the catalytic advantage conferred by the LBHB should be lost by its disruption. Accordingly, *N*-methylation of His^{57} in α-chymotrypsin decreases its acylation rate by 2×10^5 (*23*). Moreover, the overall catalytic activity of the trypsin mutant $Asp^{102} \rightarrow Asn$ (chymotrypsin numbering) was reduced to one ten-thousandth of its original value (*24*). These experiments support but do not prove the LBHB-facilitated general base mechanism.

LBHBs have been postulated to participate in the enzymatic enolization of aldehydes, ketones, and esters (*25–27*). Spectroscopic evidence for LBHBs in these enzymes has so far not been reported. It is important to verify LBHBs spectroscopically because electrostatic stabilization can also facilitate the enolization of ketones and esters (*28, 29*). The assignment of LBHBs in diverse enzymatic processes should be supported by spectroscopic information such as that obtained for serine proteases.

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Crystal Structure of Rat DNA Polymerase β: Evidence for a Common Polymerase Mechanism

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Structures of the 31-kilodalton catalytic domain of rat DNA polymerase β (pol β) and the whole 39-kilodalton enzyme were determined at 2.3 and 3.6 angstrom resolution, respectively. The 31-kilodalton domain is composed of fingers, palm, and thumb subdomains arranged to form a DNA binding channel reminiscent of the polymerase domains of the Klenow fragment of *Escherichia coli* DNA polymerase I, HIV-1 reverse transcriptase, and bacteriophage T7 RNA polymerase. The amino-terminal 8-kilodalton domain is attached to the fingers subdomain by a flexible hinge. The two invariant aspartates found in all polymerase sequences and implicated in catalytic activity have the same geometric arrangement within structurally similar but topologically distinct palms, indicating that the polymerases have maintained, or possibly re-evolved, a common nucleotidyl transfer mechanism. The location of Mn^{2+} and deoxyadenosine triphosphate in pol β confirms the role of the invariant aspartates in metal ion and deoxynucleoside triphosphate binding.

DNA polymerase β (pol β) is one of the four recognized DNA-directed DNA polymerases of the eukaryotic nucleus: α, β, δ, and ε. It has been studied primarily in vertebrates but homologs have also recently been discovered in lower eukaryotes such as yeast (*1*) and trypanosomes (*2*). Although its full physiological role has not yet been established, several lines of evidence suggest that it is an essential housekeeping enzyme (*3*): the pol β gene is highly conserved and constitutively expressed; its activity has been correlated with repair of damaged DNA; and it alone has the ability to fill single-stranded DNA gaps smaller than six nucleotides (*4*).

As a single 335-residue polypeptide chain with no associated exonuclease or proofreading activity, pol β is a relatively uncomplicated target for mechanistic studies of the nucleotidyl transfer reaction—the coupling

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