its synthesis and workup. No further scrambling was observed in the syntheses of phosphocreatinine and phosphocreatine from the [^{15}N]creatinine. A notable exception is the alternate route to the synthesis of [2/3-^{15}N]phosphocreatine. This route is the enzyme-catalyzed ^{15}N/^{14}N positional isotope exchange of [2-^{15}N]phosphocreatine by creatine kinase in the presence of MgADP. The mechanism for this scrambling of the label in phosphocreatine involves ^{15}N-labeled creatine as an intermediate.

As expected, the ^{31}P NMR data reflect an ^{15}N-induced chemical shift change of 0.01 ppm. No such change was observed in the ^{1}H resonances of protons bound to N-2 of creatinine. The line-broadening effect of the ^{15}N quadrupolar nucleus is evident in comparing the proton spectra of [2-^{15}N]creatine and [2/3-^{15}N]creatinine. Such line broadening is not apparent, however, from ^{31}P NMR spectra of the labeled phosphocreatinines and phosphocreatines.

The results of the ^{15}N and ^{1}H NMR studies reported here are consistent only with protonation exclusively at N-2 on creatinine at low pH. The evidence further suggests that the most important resonance form is that in which conjugation with the carbonyl moiety is present, although the other two forms are likely to make small contributions.

The ^{15}N and ^{31}P NMR data were used to establish unequivocally that the site of phosphorylation in phosphocreatinine is at N-2, i.e., on the exocyclic nitrogen. The phosphoryl group of phosphocreatinine thus has the opposite stereochemistry from that required for phosphoryl transfer to MgADP in the reaction catalyzed by creatine kinase. The fact that phosphocreatinine and creatinine are not substrates in the creatine kinase reaction has been interpreted to mean that the presence of a carboxyl group is necessary for substrate activity. The essential role of the carboxyl group is now called into question.

Finally, [2-^{15}N]phosphocreatine was found by this investigation to be potentially useful as a probe of the stereospecificity and kinetics of the creatine kinase reaction. Two prerequisites for this use were met. First, the ^{31}P–^{15}N one-bond coupling constant was found to be 18 Hz, not 3 Hz as reported by Brindle et al.[13] A J_{NP} value of 18 Hz is more than sufficient to allow resolution of ^{31}P NMR signals in spite of both the ^{15}N-induced chemical shift change and any line broadening induced by the ^{14}N quadrupolar nucleus. Therefore, the phosphorus resonances of [2-^{15}N]-phosphocreatine and [3-^{15}N]phosphocreatine can be resolved in order to quantitate their relative amounts in solution. Second, as stated above, the last step in the synthesis of [2-^{15}N]-phosphocreatine was not accompanied by scrambling of the label. This proves that specifically labeled phosphocreatine does not undergo ^{14}N/^{15}N positional isotope exchange all by itself.

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P_1 Residue Determines the Operation of the Catalytic Triad of Serine Proteases during Hydrolyses of Acyl-Enzymes

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Abstract: Proton inventories (rate measurements in mixtures of H_2O and D_2O) were determined for hydrolyses of acyl-enzymes that were formed during the reactions of porcine pancreatic elastase, human leukocyte elastase, and bovine pancreatic α-chymotrypsin with three peptidic substrates that differed in the P_1 amino acid residue (Ala, Val, or Phe). "Bowl-shaped" proton inventories were observed for substrates that fulfilled the enzyme's P_1 specificity requirements, while linear proton inventories were observed for P_1-nonspecific substrates. These results suggest that the P_1 residues of peptidic substrates play a critical role in determining whether the catalytic triad of serine proteases acts in a coupled manner with two-proton catalysis or simply operates as a one-proton catalyst.

The proton inventory technique[1] has been used to probe the operation of the catalytic triad[2] for a number of serine protease-catalyzed reactions.[3-9] In general, linear proton inventories, suggesting simple, one-proton catalysis by the active site histidine, have been found for minimal, nonspecific substrates, such as p-nitrophenyl acetate, while "bowl-shaped" proton inventories, consistent with two-proton catalysis and full functioning of the catalytic triad, have been found for specific substrates, such as tri- and tetrapeptide-based anilides and esters. The goal of these studies was to establish substrate structural requirements for the effective operation of the catalytic triad. Effective operation of the catalytic triad with substrate prolongation suggests a structural coupling[3] of this catalytic unit with remote protease subsites.[2] Interactions at remote subsites are known to play several important

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Table I. Hydrolysis of Thiobenzyl Esters by Serine Proteases[a]

	PPE			HLE			CT		
	k_c	K_m	k_c/K_m	k_c	K_m	k_c/K_m	k_c	K_m	k_c/K_m
Ala	38	15	2 600 000	53	13	4 100 000	6	5	1 200 000
Val	7.4	8	940 000	13	2	5 600 000	0.2	0.6	300 000
Phe	34	59	580 000	3	47	59 000	39	8	6 700 000

[a] 0.1 M HEPES, 0.5 M NaCl, pH 7.65, 3.3% Me_2SO, 25 ± 0.1 °C. Substrates: Ala, MeOSuc-Ala-Ala-Pro-Ala-SBzl; Val, MeOSuc-Ala-Ala-Pro-Val-SBzl; Phe, Suc-Ala-Ala-Pro-Phe-SBzl. Units: k_c, s^{-1}; k_m, μM; k_c/K_m, M^{-1} s^{-1}.

roles in catalysis by serine proteases.[10]

In this paper, we report that during hydrolyses of peptide-based acyl-enzymes, the P_1[11] amino acid residue plays a critical role in determining the functional integrity of the catalytic triad. We find that when P_1 specificity requirements are fulfilled, two-proton catalysis is observed, but when these requirements are not met, only single-proton catalysis is observed.

Experimental Section

Materials. HLE[13] was purchased from Elastin Products, Pacific, MO. The material was purified from purulent sputum as previously described[15] and supplied as a salt-free lyophilized powder. Preparation of stock solutions of HLE has been described.[10,15] PPE was also purchased from Elastin Products and was supplied as a salt-free, lyophilized powder. CT was from Sigma Chemical Co., St. Louis, MO. Stock solutions of PPE and CT were prepared in 10^{-3} M HCl.

MeOSuc-Ala-Pro-Ala-SBzl, MeOSuc-Ala-Ala-Pro-Ala-SBzl, and MeOSuc-Ala-Ala-Pro-Val-SBzl were available from previous studies.[14] The thiobenzyl ester and *p*-nitroanilide of Suc-Ala-Ala-Pro-Phe were purchased from Bachem, Inc. (Torrace, CA) and Sigma Chemical Co., respectively. MeOSuc-Ala-Ala-Pro-Val-CH_2Cl was available from a previous study.[16] Buffer salts and Me_2SO were reagent grade from several suppliers. D_2O (99.8 atom % D) was from Sigma Chemical Co.

Kinetic Methods. Methods for monitoring the hydrolyses of thiobenzyl esters and calculation of kinetic constants have been described,[14] as have the methods for the determination of solvent isotope effects and proton inventories.[3,4]

Inactivation of Protease Activity by MeOSuc-Ala-Ala-Pro-Val-CH_2Cl. Thirty microliters of a 1.0-mg/mL stock solution of CT was added to 100 mL of pH 7.6 buffer (0.1 M HEPES, 0.5 M NaCl). To this solution was added 100 μL of a 10 mM stock solution (Me_2SO) of MeOSuc-Ala-Ala-Pro-Val-CH_2Cl. The final CT and chloromethyl ketone concentrations were 10 nM and 10 μM, respectively. At several predetermined times, two 3-mL aliquots of the reaction solution were removed and each added to a cuvette. To one cuvette was added MeOSuc-Ala-Ala-Pro-Ala-SBz ($[S]_0$ = 69 μM) while to another Suc-Ala-Ala-Pro-Phe-pNA ($[S]_0$ = 167 μM) was added. Steady-state velocities of product release were determined. These velocities are proportional to the residual enzyme concentration and when plotted on semilog paper vs. time provide first-order inactivation rate constants.

Results

Purity of Protease Solutions. Since the object of this investigation was to study the P_1 specificity of deacylation, it was essential that our protease preparations be free of small amounts of contaminating proteases. To determine if our CT solutions were contaminated with elastase, we conducted an experiment in which the solution was incubated with the chloromethyl ketone MeO-Suc-Ala-Ala-Pro-Val-CH_2Cl, a known inactivator of elastase.[16,17] Activity toward MeOSuc-Ala-Ala-Pro-Ala-SBzl, an elastase-specific substrate,[14] and Suc-Ala-Ala-Pro-Phe-pNA, a substrate with known specificity for chymotrypsin-like enzymes, disappeared with identical first-order rate constants of 5.8×10^{-5} s^{-1}. This result suggests that a single enzyme, able to hydrolyze both substrates, was being inactivated. If this rate constant is divided by the chymotrypsin concentration (13 nM), a second-order rate constant of 0.17 M^{-1} s^{-1} is obtained. This value is equivalent to the inactivation parameter k_i/K_i and should be compared with a value of k_i/K_i of 580 M^{-1} s^{-1} determined for the inactivation of PPE by this chloromethyl ketone.[17] Combined, these results indicate the following: (1) There is no elastase in the CT preparation that was used in these experiments. (2) Hydrolytic activity toward Suc-Ala-Ala-Pro-Phe-pNA, as well as toward MeOSuc-Ala-Ala-Pro-Ala-SBzl, is due to catalysis by CT. (3) CT can be inactivated, albeit very sowly, by MeOSuc-Ala-Ala-Pro-Val-CH_2Cl.

The porcine elastase used in this study is a high-purity product from Elastin Products. It is twice-crystallized and then chromatographed on DEAE-cellulose at pH 8.8 and on CM-cellulose at pH 5.0. Trace trypsin contamination is removed by affinity chromatography. The final product is homogeneous on 15% polyacrylamide electrophoresis at pH 4.5 and on gel exclusion chromatography (G200) at pH 5.0. There is no detectable trypsin or chymotrypsin activity with the specific substrates, *N*-benzoyl-DL-arginine-pNA and *N*-benzoyl-L-tyrosine ethyl ester, respectively.

The purification and purity of the human leukocyte elastase that was used in these studies have been reported.[15]

P_1 Specificity of Serine Proteases. Table I contains steady-state kinetic parameters for the hydrolyses of MeOSuc-Ala-Ala-Pro-Ala-SBzl, MeOSuc-Ala-Ala-Pro-Val-SBzl, and Suc-Ala-Ala-Pro-Phe-SBzl by porcine pancreatic elastase, human leukocyte elastase, and bovine pancreatic α-chymotrypsin. Kinetic parameters were also determined for the PPE-catalyzed hydrolysis of MeOSuc-Ala-Pro-Ala-SBzl: k_c = 47 s^{-1}, K_m = 31 μM, k_c/K_m = 1 200 000 M^{-1} s^{-1}.

The P_1 specificity that is reflected in the data of Table I is broader than we anticipated. Kinetic results for the hydrolyses of anilides and amides[18] as well as thiobenzyl esters of the general form Boc-Ala-Ala-X-SBzl[19] indicate much narrower specificities for all three proteases. Interestingly, the breadth of specificity we observe is expressed not only during acylation but also during deacylation. However, the acylation results are not too surprising in light of the known reactivity of thiobenzyl esters toward serine proteases.

Proton Inventories of Deacylation. Proton inventories of k_c were determined for the following serine protease reactions: PPE, MeOSuc-Ala-Pro-Ala-SBzl; PPE, MeOSuc-Ala-Ala-Pro-Ala-SBzl; PPE, MeOSuc-Ala-Ala-Pro-Val-SBzl; PPE, Suc-Ala-Ala-Pro-Phe-SBzl; HLE, Suc-Ala-Ala-Pro-Phe-SBzl; CT, MeOSuc-Ala-Ala-Pro-Ala-SBzl; CT, MeOSuc-Ala-Ala-Pro-Val-SBzl; and CT, Suc-Ala-Ala-Pro-Phe-SBzl (proton inventory data and details of analyses can be obtained as supplementary material to this paper). Since k_c for the hydrolyses of thiobenzyl esters is equal

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Table II. Proton Inventories for the Hydrolyses of Acyl-Enzymes of Serine Proteases[a]

acyl enzyme	k_3 (s^{-1})	${}^{D}k_3$	no. of active protons	ϕ_1	ϕ_2
MeOSuc-Ala-Pro-Ala-PPE	47	3.08 ± 0.03	2	0.570	0.570
MeOSuc-Ala-Ala-Pro-Ala-PPE	38	2.64 ± 0.12	1	0.379	1.000
Suc-Ala-Ala-Pro-Phe-PPE	34	2.41 ± 0.13	1	0.415	1.000
MeOSuc-Ala-Ala-Pro-Ala-HLE[b]	53	3.56 ± 0.14	2	0.53	0.53
MeOSuc-Ala-Ala-Pro-Val-HLE[b]	13	2.84 ± 0.07	2	0.593	0.593
Suc-Ala-Ala-Pro-Phe-HLE	3	2.52 ± 0.07	1	0.397	1.000
MeOSuc-Ala-Ala-Pro-Ala-CT	6	2.79 ± 0.05	1	0.358	1.000
MeOSuc-Ala-Ala-Pro-Val-CT	0.2	3.17 ± 0.14	1	0.315	1.000
Suc-Ala-Ala-Pro-Phe-CT	56	2.44 ± 0.07	2	0.640	0.640

[a] 0.1 M HEPES, 0.5 M NaCl, pH 7.65 and pD equivalent, 3.3% Me_2SO, 25 ± 0.1 °C. Proton inventories were constructed from replicate rate determinations (n = 3–5) at 6 to 11 mole fractions of solvent deuterium and analyzed according to eq 1 with the constraint that either $\phi_1 = \phi_2$ (2 active protons) or $\phi_2 = 1.0$ (1 active proton). Average error in $k_{n=0}/k_{n=1}$ ranged from 1.5% to 3.6%; average residual deviation from the best-fit line ranged from 0.9% to 4.3%. The isotope effects and fractionation factors of this table are based on the analyses of 2 to 4 separate proton inventory experiments. [b] See ref 3.

to the first-order rate constant for deacylation,[14] k_3, these proton inventories are for acyl-enzyme hydrolysis.

For the deacylation of MeOSuc-Ala-Pro-Ala-PPE and Suc-Ala-Ala-Pro-Phe-CT, the proton inventories are bowed downward and suggest that the data conform the eq 1. Equation 1 is a

$$k_{3,n}/k_{3,n=0} = (1 - n + n\phi_{T1})(1 - n + n\phi_{T2}) \quad (1)$$

simplification of the more general Gorss–Butler eq 1 and states that the observed solvent isotope effect on deacylation is generated by deuterium fractionation at two exchangeable sites. In this equation, n is the mole fraction of solvent deuterium, and ϕ_{T1} and ϕ_{T2} are transition-state fractionation factors, relative to bulk water, for the two isotope effect generating protonic sites. The data for these proton inventories were fit to eq 1 with the constraint that $\phi_{T1} = \phi_{T2}$. Fitting the data to eq 1 without constraints invariably yielded values for ϕ_{T1} and ϕ_{T2} that differed from each other by less than 1% (and thus equal the fractionation factor obtained by fittng the data with $\phi_{T1} = \phi_{T2}$) but had unrealistically large standard errors.

In contrast, for the deacylation of MeOSuc-Ala-Ala-Pro-Ala-PPE, Suc-Ala-Ala-Pro-Phe-PPE, Suc-Ala-Ala-Pro-Phe-HLE, MeOSuc-Ala-Ala-Pro-Ala-CT, and MeOSuc-Ala-Ala-Pro-Val-CT, the proton inventories were found to be linear and suggest one-proton catalysis. These data were fit to eq 1 with ϕ_{T2} constrained to 1.0. The results of these analyses are summarized in Table II.

Also in Table II are the results of previously determined proton inventories for the hydrolyses of MeOSuc-Ala-Ala-Pro-Ala-HLE and MeOSuc-Ala-Ala-Pro-Val-HLE.[3]

Discussion

Previous studies[1,3-10] indicate that the number of protonic sites involved during catalysis by serine proteases is dependent on the length of the substrate and thus on the number of protease subsites with which the substrate interacts. Results from several of these studies for the deacylation of PPE, HLE, and CT are summarized in Table III and suggest a positive correlation between the number of occupied subsites and the number of protonic sites of the catalytic triad that are activated in the transition state. We see that when substrates are elongated such that they are able to interact with subsites at and beyond S_4, the catalytic triad is fully activated, and deacylation occurs with the transfer of two protons.

In this study, we set out to determine if another substrate structural feature can influence coupling of the catalytic triad. We found that the P_1 residue plays a critical role in determining the functional integrity of the catalytic triad. For HLE and CT, changing the P_1 residue of specific tetrapeptide substrates to nonspecific residues uncouples the catalytic triad and, in so doing, reduces the triad to a simple, one-proton catalyst. For PPE, however, we observe a more complex situation.

Hydrolysis of the tripeptide-based acyl enzyme MeOSuc-Ala-Pro-Ala-PPE occurs with two-proton catalysis. This is consistent with the results of Hunkapillar, Forgac, and Richards,[8] who observed a bowed-downward proton inventory of k_2 for the PPE-catalyzed hydrolysis of Ac-Ala-Pro-Ala-pNA. As we, they interpreted their results to suggest two-proton catalysis and operation of the catalytic triad. In sharp contrast to both of these results, we observed a *linear* proton inventory for the hydrolysis of the tetrapeptide-based acyl-enzyme, MeOSuc-Ala-Ala-Pro-Ala-PPE. A linear proton inventory such as this is usually interpreted to suggest a mechanism involving one-proton catalysis and an uncoupled catalytic triad. This observation runs contrary to expectations for a P_1-specific, tetrapeptide-based acyl-enzyme[1,3-10] and therefore may justify the proposal of alternate mechanistic explanations.

Table III. Dependence of Coupling of the Catalytic Triad during Deacylation of Serine Proteases on Substrate Length

acyl enzyme	solvent isotope effect	no. of active protons	ref
Ac-PPE	2.45	1	7
Z-Gly-PPE	2.45	1	5
MeOSuc-Ala-Pro-Ala-PPE	3.08	2	*a*
MeOSuc-Val-HLE	2.72	1	3
MeOSuc-Pro-Val-HLE	3.22	1	3
MeOSuc-Ala-Pro-Val-HLE	3.08	2	3
MeOSuc-Ala-Ala-Pro-Val-HLE	2.87	2	3
Ac-CT	2.45	1	9
Z-Trp-CT	2.45	1	1
Z-Phe-CT	2.45	1	1
Suc-Ala-Ala-Pro-Phe-CT	2.44	2	*a*

[a] This work.

One such explanation involves a mechanism in which k_3 is only *partially* rate-limited by the hydrolytic events of deacylation. A linear proton inventory can easily be obtained from a model involving partial rate-limitation by two steps: one involving two-proton acyl-enzyme hydrolysis and the other some non-chemical step that generates no isotope effect.[20] Although speculative, this mechanism is consistent with kinetic data for PPE. k_3 for the hydrolysis of MeOSuc-Ala-Ala-Pro-Ala-PPE (38 ± 2 s^{-1}) is significantly smaller than k_3 for the hydrolysis of MeOSuc-Ala-Pro-Ala-PPE (47 ± 2 s^{-1}), and both values are much smaller than the rate constant of 300 s^{-1} for the hydrolysis of Ac-Ala-Pro-Ala-PPE.[8] These results suggest that interactions

(20) The proton inventory for the hydrolysis of MeOSuc-Ala-Ala-Pro-Ala-HLE can be fit to the equation

$$k_{3,n}/k_{3,n=0} = \left[C_1 + \frac{C_2}{(1 - n + n\phi)^2} \right]^{-1}$$

in which C_1 and C_2 are the fractional contributions of the two partially rate-limiting steps [see ref 21 and 22 for a full explanation of this model]. According to this fit, $C_1 = 0.28 \pm 0.15$ ($C_2 = 1 - C_1$) and $\phi = 0.540 \pm 0.048$. The sum of the square of the residuals is 0.046 (F = 3644) and should be compared with the sum of the square of residuals for the linear model of 0.044 (F = 7735).

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of an N-terminus blocking group at S_4 through S_6 can have major effects on catalytic efficiency and rate-limiting steps.

Together with previous studies,[1,3–10] the results of this investigation indicate that substrate structural features regulate an important component of the catalytic apparatus possessed by serine proteases: the coupling and effective operation of the catalytic triad. This may represent an example of a more general phenomenon in which interactions between the enzyme and nonreacting portions of the substrate not only pay the energetic cost of catalysis[23] but also enforce one of several mechanistic alternatives available to the enzyme.[3,10,24]

Supplementary Material Available: Table giving the analysis of proton inventories for the hydrolyses of acyl-enzymes of serine proteases and figures showing the 16 proton inventories discussed in the text (18 pages). Ordering information is given on any current masthead page.

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Communications to the Editor

Studies on Tumor Promoters.[1] The First Synthesis of the Phorbol Skeleton

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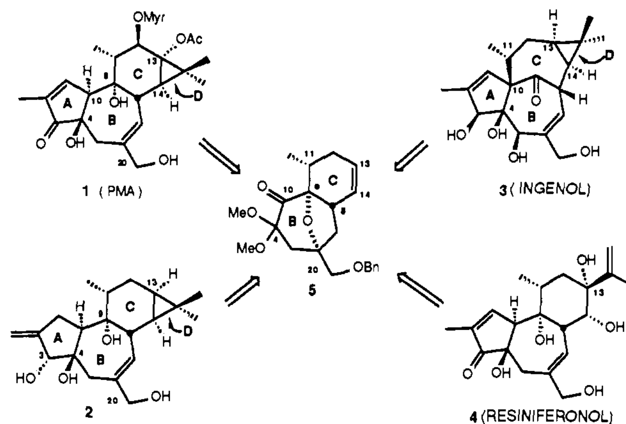
The mechanism of carcinogenesis has been the focus of global research for most of this century.[3] The early view of a unifactorial relationship between carcinogen and tumor formation was expanded to accommodate a multistage mechanism in the 1930s when it was demonstrated that several noncarcinogenic compounds, now referred to as tumor promoters, amplified the effect of carcinogens on mouse skin. The most potent of these compounds was later shown to be phorbol myristate acetate (**1**, PMA; Scheme I).[3] More recently, the major PMA receptor was identified as protein kinase C (PKC)[4]—a ubiquitous enzyme of great organomedicinal interest because of its critical role in hormonal signal transduction in cells. We describe herein the first synthesis of a stereochemically complete tigliane skeleton in the form of phorboid **2**. This compound is a designed hybrid of the tigliane and ingenane promoters which was formulated to probe systematically our recently proposed pharmacophore model for PKC activation[1a] and to define the structural requirements for diterpene recognition at the PKC regulatory domain.[1,5]

Our program goals necessitated the development of a divergent synthetic strategy in order to service studies on the tigliane, ingenane, and daphnane promoters (**1**, **3**, and **4**, respectively).[6] The strategy which best met this objective called for the construction of a BC ring system (e.g., **5**) common to two families (**1** and **4**) and convertible to the third (**3**) through rearrangement (C11 to C10). Subsequent attachment of the A ring to C4 and C10 in **5** and the D ring to C13 and C14 or an isopropenyl to C13 would then provide access to all three skeleta. The seemingly superfluous oxygen bridge in **5** was incorporated in this plan to provide internal protection of the C-9 oxygen and to convert the otherwise flexible seven-membered B-ring and cyclohexenyl C-ring into a conformationally and facially biased tricyclic system capable of guiding the genesis of key stereocenters. A further attribute of this plan was the potential ready availability of the key intermediate **5** through the intramolecular Diels–Alder reaction of the triene **9** (Scheme II).

Scheme I



The execution of the above strategy, initially entailing the synthesis of **9**, started with the hetero-Diels–Alder reaction of 2-methoxybutadiene[7a] and ethyl glyoxalate,[7b] from which only one pyran product was obtained (**6**,[8] 60%). Introduction of the diene

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