correlations is supported by the following observations: (i) the standard synthetic procedures produce atypically small yields of these complexes (see Experimental Section); (ii) alkaline alkyl–Co bond cleavage rates for the monohalomethylcobaloximes are orders of magnitude faster than those for all other alkylcobaloximes;[17] and (iii) the ligand association and dissociation rate constants for these complexes are significantly larger than those of other alkyl derivatives as is expected if dissociative mechanisms are in play[30b] (see below) for sterically hindered complexes. Inspection of the covalent radii for these halogens (0.99 Å for Cl, 1.33 Å for I, compared with 0.32 Å for H in CH_4)[31] shows that the size of these substituents is approaching the lengths of the carbon–cobalt bonds which run from about 1.94 to 2.05 Å for numerous alkylcobalt complexes.[32-36] (b) F. Basolo and R. G. Pearson, "Mechanisms of Inorganic Reactions", 2nd ed, Wiley, New York, N.Y., 1967, p 387.

(31) L. Pauling, "The Nature of the Chemical Bond", 3rd ed, Cornell University Press, New York, N.Y., 1966, p 224.
(32) (a) S. Brückner, M. Calligaris, G. Nardin, and L. Randaccio, *Inorg. Chim. Acta*, **3**, 308 (1969); (b) M. Calligaris, G. Nardin, and L. Randaccio, *Inorg. Nucl. Chem. Lett.*, **8**, 477 (1972).
(33) S. Brückner, M. Calligaris, G. Nardin, and L. Randaccio, *Inorg. Chim. Acta*, **2**, 416 (1968).
(34) P. G. Lenhert, *Chem. Commun.*, 980 (1967).
(35) M. Calligaris, D. Minichelli, G. Nardin, and L. Randaccio, *J. Chem. Soc., A*, 2720 (1971).
(36) M. Calligaris, G. Nardin, and L. Randaccio, *J. Chem. Soc., Dalton Trans.*, 1433 (1972).
(37) G. Costa, *Pure Appl. Chem.*, **30**, 335 (1972).
(38) (a) G. N. Schrauzer, L. P. Lee, and J. W. Sibert, *J. Am. Chem. Soc.*, **92**, 2997 (1970); (b) H. A. O. Hill, J. M. Pratt, and R. J. P. Williams, *Discuss. Faraday Soc.*, **47**, 165 (1969).
(39) R. A. Firth, H. A. O. Hill, B. E. Mann, J. M. Pratt, and R. G. Thorp, *Chem. Commun.*, 1013 (1967).
(40) H. A. O. Hill, J. M. Pratt, and R. J. P. Williams, *Chem. Br.*, **5**, 156 (1969).
(41) R. A. Firth, H. A. O. Hill, B. E. Mann, J. M. Pratt, R. G. Thorp, and R. J. P. Williams, *J. Chem. Soc. A*, 2419 (1968).
(42) A. Bigotto, G. Costa, G. Mestroni, G. Pellizer, A. Puxeddu, E. Reisenhofer, L. Stefani, and G. Tauzher, *Inorg. Chim. Acta Rev.*, **41**, 4 (1970).
(43) G. Costa, G. Mestroni, G. Tauzher, D. M. Goddall, and H. A. O. Hill, *Chem. Commun.*, 34 (1970).
(44) G. Costa, G. Mestroni, G. Tauzher, and L. Stefani, *J. Organomet. Chem.*, **6**, 181 (1966); G. Costa and G. Mestroni, *ibid.*, **11**, 325 (1968); G. Costa, G. Mestroni, and L. Stefani, *ibid.*, **7**, 493 (1967); G. Costa, G. Mestroni, and G. Pellizer, *ibid.*, **11**, 333 (1968).
(45) C. Giannotti, B. Septe, and D. Benlian, *J. Organomet. Chem.*, **39**, 5c (1972).
(46) G. Costa, G. Tauzher, and A. Puxeddu, *Inorg. Chim. Acta*, **3**, 41 (1969).
(47) G. N. Schrauzer and R. J. Windgassen, *J. Am. Chem. Soc.*, **88**, 3738 (1966).
(48) L. M. Ludwick, and T. L. Brown, *J. Am. Chem. Soc.*, **91**, 5188 (1969).
(49) T. L. Brown, L. M. Ludwick, and R. S. Stewart, *J. Am. Chem. Soc.*, **94**, 384 (1972).
(50) For ligation by L in a system in which dimers (D) of the alkylcobaloxime monomers (M) occur

$$D \underset{K_d}{\rightleftharpoons} M + M \overset{K_x}{\underset{\pm L}{\rightleftharpoons}} ML$$

the apparent equilibrium constant for ligand association (K_l) is given by

$$K_l = \frac{2K_x}{1 - b \pm (b^2 + 2K_d C_T)^{1/2}}$$

where $K_d = [M]^2/[D]$, $K_x = [ML]/[M][L]$, $b = (1 + K_x[L])/2$, and C_T is the total alkylcobaloxime concentration. The K_l values therefore depend upon the total alkylcobaloxime concentrations employed.
(51) A. V. Ablov and M. P. Filippov, *Zh. Neorg. Khim.*, **5**, 2717 (1960).
(52) B. A. Borykin, *Zh. Neorg. Khim.*, 17, 169 (1972).
(53) G. P. Syrtsova, *Zh. Neorg. Khim.*, 17, 172 (1972).
(54) J. E. Earley and J. G. Zimmerman, *Inorg. Nucl. Chem. Lett.*, **8**, 687 (1972).
(55) J. Zsako, Z. Finta, and C. S. Varhelyi, *J. Inorg. Nucl. Chem.*, **34**, 2887 (1972).
(56) G. Tauzher, R. Dreos, and G. Costa, *J. Chem. Soc., Chem. Commun.*, 413 (1973).
(57) T. Sakurai, J. P. Fox, and L. L. Ingraham, *Inorg. Chem.*, **10**, 1105 (1971).
(58) D. N. Hague and J. Halpern, *Inorg. Chem.*, **6**, 2059 (1967).
(59) R. B. Silverman, D. Dolphin, and B. M. Babior, *J. Am. Chem. Soc.*, **94**, 4028 (1972).
(60) H. A. O. Hill, K. G. Morallee, F. Cernivez, and G. Pellizer, *J. Am. Chem. Soc.*, **94**, 277 (1972).
(61) An exact treatment of the kinetics of SN1 mechanism of eq 5a with regard to the dependence of the observed k_{on} values upon pyridine concentration under circumstances in which "saturation kinetics" is observed will be included in a future publication. In this instance, "saturation kinetics" is the term applied to concave downward plots of k_{on} vs. [L] i.e., the progression from first-order to zero-order dependence upon the concentration of [L] of k_{on}. Such "saturation kinetics" reflect a change in rate-determining step from ligation of pentacoordinate alkylcobaloxime by L, k_2, to rate-determining dehydration of the RCo(D_2H_2)(HOH) species, k_1 (eq 5a): P. S. Tobias and R. G. Kallen, unpublished observations.

Substrate Induced p*K* Perturbations with Chymotrypsin and the Possible Significance of Nonproductive Binding[1]

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Abstract: The binding of the substrates *N*-acetyl-L-tyrosine *p*-chloroanilide and *N*-formyl-L-phenylalanine formylhydrazide to chymotrypsin is accompanied by a significant perturbation of the p*K* of a group on the enzyme. This perturbation does not occur with the closely related nonsubstrate derivatives *N*-formyl-D-phenylalanine formylhydrazide, *N*-formyl-*N*-methyl-L-phenylalanine formylhydrazide, *N*-acetyl-D-tyrosine *p*-chloroanilide, and *N*-acetyl-*N*-methyl-L-tyrosine *p*-chloroanilide. These results are consistent with a hypothesis that a component of the catalytic process can contribute to the binding of substrates. The recent suggestion that the nature of the aniline moiety in acylamino acid anilide substrates influences the apparent binding (K_m) by contributing to nonproductive binding could not be substantiated. It was found that at pH 7.13 *N*-acetyl-D-tyrosine *p*-chloroanilide, a compound which is especially suited for the proposed nonproductive binding mode, is not bound as well as is the corresponding L substrate. The rate of reaction of formylphenylalanylchymotrypsin with formylhydrazine and of acetyltyrosylchymotrypsin with *p*-chloroaniline and ammonia has the same pH dependence as that for hydrolysis of these acyl enzymes. These results rule out a previously proposed mechanism in which there is a pH-induced change in rate-determining step. The results are also not in accord with a mechanism suggested to account for the pH-dependent ^{15}N isotope effect seen in the hydrolysis of acetyl-L-tryptophan amide.

It has been found with chymotrypsin that with a number of substrates with a weakly basic amine leaving group the K_m is pH dependent in the neutral pH range.[2] To determine whether this behavior is a reflection of the binding process being influenced by the pH-dependent catalysis or by pH-dependent nonproductive binding, we have studied the pH dependence of the binding of nonsubstrate analogues of the compounds for which the unusual pH effect is observed. We also report results bearing on a proposed change in rate-determining step in the catalytic mechanism.[2c,3]

Results

Apparent dissociation constants were measured by determining the degree of inhibition of the hydrolysis of *p*-nitrophenyl acetylglycinate, methyl hippurate, or HCO-Phe-FH.[5] Equation 1, a modified form of the usual competitive

$$K_I = \frac{I}{(K' + 1 + S/K_m)[(v/v_i) - 1]} \quad (1)$$

inhibition expression, has been derived for Scheme I. E′ and E are inactive and active conformations of the enzyme, re-

Scheme I

$$E' \overset{K'}{\rightleftharpoons} E \overset{K_m}{\rightleftharpoons} ES$$
$$\Big\Updownarrow K_I$$
$$EI$$

spectively;[4] I is the inhibitor; S is the substrate; v and v_i are uninhibited and inhibited initial rates respectively; $K' = E'/E$; $K_m = (E)(S)/(ES)$; and $K_I = (E)(I)/(EI)$. The so-determined apparent dissociation constants are given in Table I. The constants obtained with Ac-Tyr-NHPhCl[5] are in excellent agreement with those determined by kinetic study.[2b]

Values of K_m for *p*-nitrophenyl acetylglycinate were determined under the conditions of each reaction by a weighted least-squares program[6] of a double reciprocal plot. These were corrected for the fraction of enzyme present in the active form[4] and are given in Table II. The K_m's for HCO-Phe-FH[2d] and for methyl hippurate[7] were previously published.

The inhibition of chymotrypsin by Ac-MeTyr-NHPhCl[5] is time dependent at pH 6.31: with 1.2 m*M* inhibitor the apparent K_I is 4 m*M* when the inhibitor is preincubated with the enzyme for 70 min, as compared with 13 m*M* without preincubation. With 1.2 m*M* inhibitor this corresponds to a change in the degree of inhibition from 4.7 (zero time) to 13.9% (70 min). No such time dependence was observed at pH 7.54 with this inhibitor, and the inhibition by HCO-MePhe-FH[5] is not time dependent at any pH studied. Full reversal of the inhibition by Ac-MeTyr-NHPhCl is not instantaneous; after a tenfold dilution of an aliquot of a 70-min aged enzyme mixture containing 1.2 m*M* inhibitor into a solution of 1.2 m*M* inhibitor for assay of activity, the enzyme was found to be 13.9% inhibited; when the dilution was done into an assay mixture not containing inhibitor the enzyme was 4.7% inhibited. The inhibition in the 0.12 m*M* inhibitor mixture was greater than the 1.6% inhibition calculated from the K_I determined with 1.2 m*M* inhibitor. No enzymic hydrolysis of Ac-MeTyr-NHPhCl was found to occur with overnight incubation.

Rates of acetyltyrosyl- or formylphenylalanylchymotrypsin aminolysis were determined by product analysis. In reactions of water and an amine with an acyl enzyme under conditions where no significant fraction of the enzyme exists as an amine–enzyme complex, the product ratio is determined by the rate constants and amine concentration as defined in the relationship

$$\frac{\text{yield of amide}}{\text{yield of carboxylic acid}} = \frac{k_{\text{amine}}[\text{amine}]}{k_{H_2O}} \quad (2)$$

In the reaction of acetyltyrosyl- and formylphenylalanylchymotrypsin, the rate constants for acyl enzyme hydrolysis are 193 and 85 sec^{-1}, respectively.[8]

The so-determined rate constants are summarized in Table III. For acetyltyrosylchymotrypsin the partitioning with water and *p*-choloroaniline is pH invariant over the range 5.5–7.5. For formylphenylalanylchymotrypsin the partitioning with water and formylhydrazine is pH invariant over the range 4.5–7.5.

Table I. Apparent Dissociation Constants for α-Chymotrypsin[a]

Amide	Amide concn, m*M*	pH	K_I,[b,c] m*M*
N-Formyl-L-phenylalanine amide	22–30	7.05	37 ± 4[d]
	22–30	6.10	28 ± 3[d]
N-Formyl-D-phenylalanine amide	22–30	7.05	10 ± 1[d]
	22–30	6.10	14 ± 3[d]
N-Formyl-L-phenylalanine formylhydrazide[h]		7.30	2.6
		5.50	8.2
		4.50	14.2
N-Formyl-D-phenylalanine formylhydrazide	3.3–6.6	7.50	5.8 ± 0.9[e]
	5.6–8.5	7.50	5.4 ± 1.1[f]
	7.2–10.3	5.42	8.4 ± 1.3[f]
	7.7	4.40	>20[f]
N-Formyl-*N*-methyl-L-phenylalanine formylhydrazide	4.4–8.8	7.50	7.2 ± 1.7[e]
	5.3–8.6	7.50	11.8 ± 2.5[f]
	3.5	5.42	5.6 ± 0.4[f]
	6.5–7.2	4.40	>20[f]
N-Acetyl-L-tyrosine *p*-chloroanilide	1.0–1.5	7.13	0.83 ± 0.17[d]
	1.0	5.98	3.0 ± 0.1[d]
	1.1	5.88	>5[d]
N-Acetyl-D-tyrosine *p*-chloroanilide	1.1–1.6	7.13	1.8 ± 0.4[d]
	1.1	5.98	2.3 ± 0.2[d]
	1.4	5.88	2.6 ± 0.4[d]
N-Acetyl-*N*-methyl-L-tyrosine *p*-chloroanilide	1.2	7.54	2.7 ± 1.4[d], 2.9 ± 0.4[d,g]
	1.2–1.3	6.31	>13,[d] 4.0 ± 0.3[d,g]

[a] Reactions at 25°, with 0.05–0.07 *M* phosphate buffer for the studies with *p*-nitrophenyl acetylglycinate. The enzyme concentration was 1–4 μ*M*. The ionic strength was maintained with KCl at 0.10 for all reactions except those with Ac-Tyr-NHPhCl and Ac-D-Tyr-NHPhCl, where the ionic strength was maintained at 0.16 with KCl. The studies with the chloroanilides were carried out in 5% dimethylformamide; the reactions with *N*-formyl-D- and L-phenylalanine amide were done in 5% dimethyl sulfoxide; the reactions with the formylhydrazides were carried out in water. [b] Reported as average ± standard deviation for 2–16 determinations. [c] Corrected for the fraction of the enzyme present in the active form.[4] [d] Results obtained with the *p*-nitrophenyl acetylglycinate assay. [e] Results obtained with the methyl hippurate assay. [f] Results obtained with the HCO-Phe-FH assay. [g] Results determined after 70-min preincubation of enzyme and inhibitor. [h] Results from ref 2d.

Table II. K_m for *p*-Nitrophenyl Acetylglycinate[a]

pH	Solvent	K_m,[b] μ*M*
6.10	5% dimethyl sulfoxide	33
7.05	5% dimethyl sulfoxide	41
6.93	5% dimethylformamide	69
6.31	5% dimethylformamide	71
5.36	5% dimethylformamide	60

[a] Reactions were run at 25°, with 0.05–0.07 *M* phosphate buffer and the ionic strength was maintained at 0.1 with KCl, except for the reaction at pH 5.36 where the ionic strength was 0.16. The enzyme concentration was $1–3 \times 10^{-6}$ *M*. [b] Corrected for the fraction of the enzyme present in the active form.[4]

Discussion

A Mechanism for Perturbing the Histidine-57 p*K*. The substrate-induced perturbation of the Histidine-57 p*K* is reflected in an unusually low p*K* influencing V_{max} and a pH dependence for K_m in the neutral pH range. This effect has been accounted for,[2a,d] on the basis of the substrate specificity of the phenomenon, by assuming that the p*K* perturbation occurs in an intermediate which is formed and can accumulate in the conversion of the Michaelis complex to the acyl enzyme. The proposed intermediate is presumed to

Table III. Partitioning of Acetyltyrosylchymotrypsin between Water and *p*-Chloroaniline and of Formylphenylalanylchymotrypsin between Water and Formylhydrazine[a]

pH	*p*-Chloroaniline,[b] *M*	Formylhydrazine,[b] *M*	Enzyme, $M \times 10^6$	% amide formed	k_{amine}/k_{H_2O}
7.5	0.0460		2.3	0.550	0.121
7.5	0.0326		2.5	0.364	0.112
6.5	0.0487		2.5	0.625	0.129
6.5	0.0326		2.5	0.370	0.114
5.5	0.0487		24	0.668	0.138
5.5	0.0326		24	0.396	0.122
7.5		0.0505	0.1	5.76	1.21
7.5		0.101	0.1	10.5	1.16
6.0		0.0505	4	5.56	1.17
6.0		0.101	4	10.7	1.19
4.5		0.0493	100	5.44	1.17
4.5		0.0986	100	10.2	1.17

[a] Reactions at 25°, in 0.1 *M* KCl (with 10% dimethylformamide for the reaction of *p*-chloroaniline). Acetyltyrosylchymotrypsin was generated with 1.6 m*M* *N*-[*carboxyl*-^{14}C]acetyltyrosine methyl ester (7×10^5 cpm/μmol) and the formylphenylalanyl enzyme with 0.7 m*M* *N*-formyl[U-^{14}C]-L-phenylalanine methyl ester (2.18×10^5 cpm/μmol). [b] Concentration of the amine free base.

be a tetrahedral adduct formed by reaction of the active-site serine residue with the susceptible bond of the substrate. Although tetrahedral intermediates are ordinarily extremely unstable even in intramolecular reactions,[9] this might well occur in the enzymatic process. Support of this is provided by the x-ray crystallographic analysis of trypsin–trypsin inhibitor complexes in which it is found that the enzyme's active site serine has added to a peptide bond of the inhibitor to form a stable isolable tetrahedral adduct.[10] The fact that the p*K* perturbation is only seen with substrates containing an electron-deficient amine leaving group can be taken to indicate that electron withdrawal favors the equilibrium for formation of the putative intermediate with perturbed p*K*.

For the proposed mechanism it is predicted that factors which disrupt catalysis and formation of the intermediate will uncouple the effect of the tetrahedral intermediate-forming equilibrium from the apparent binding (K_m). Thus, it is expected that substances which are not susceptible to cleavage by the enzyme will not manifest the characteristics which have been taken as evidence for a contribution of the tetrahedral intermediate-forming equilibrium to the K_m: an unusually small K_m at alkaline pH's and the p*K* perturbation which influences both V_{max} and K_m. Also, since this equilibrium is presumed to be significant only for amide substrates with electron-withdrawing amino substituents, electron-rich amides are expected to behave similarly to unreactive substrate analogues. These predictions are confirmed by the results reported here.

As required, the binding of HCO-Phe-NH_2[5] and HCO-D-Phe-NH_2[5] (Table I), which contain a relatively electron-rich amino function, is comparatively weak and pH invariant. Also, the affinity of the L-amide is significantly less than that of HCO-Phe-FH,[2d] and the binding is not influenced by the ionization of Histidine-57. The somewhat tighter binding seen with the D-amide, as compared with the L-amide, parallels results seen with eight other related amides.[11] This intriguing relationship presumably reflects the ability of the D isomer to bind in an especially tight nonproductive binding mode in which the amino acid side chain and the acylamino group are correctly located, the α proton is in the leaving group site, and the leaving group is in the locus designated for the α proton.[11]

The higher affinity of the unsubstituted D-amides makes the observed weaker binding of HCO-D-Phe-FH,[5] as compared with the L isomer, especially interesting. In accord with the hypothesis that the tetrahedral intermediate-forming equilibrium plays no role in the binding of the D isomer, K_1 is not significantly influenced by protonation of the active site Histidine-57. Although it was suggested[12] that the decreased binding seen with the L substrate at very low pH's[2c,d] is caused by enzyme polymerization, the enzyme concentrations used[2d] were below those which were shown to polymerize. The low pH effect on the binding of the D inhibitor and L substrate is as yet inexplicable.

It was previously found with acetyl-L-tyrosine methyl ester[13] and acetyl-L-tyrosinehydroxamic acid[14] that replacement of the acylamino proton by a methyl group has no significant effect on K_m, although the V_{max} values are decreased 10^5- and 10^3-fold, respectively. In contrast with these results it is seen that replacement of the acylamino proton in HCO-Phe-FH has a substantial effect on binding. This and the different pH dependence for the binding of HCO-MePhe-FH and HCO-Phe-FH (Table I) suggest that an element contributing to the binding of the substrate is not available with the inhibitor.

Although the results reported here are in accord with the requirements of the proposed mechanism,[2a,d] it remains unproven in light of the failure to obtain direct evidence for the accumulation of an intermediate.[2c] All that can be conclusively derived from the results reported here is that some element which contributes to the binding of substrates is unable to contribute to the binding of inhibitors.[2c]

Analysis of Alternate Mechanisms. It has been suggested that the structure–reactivity studies with anilide substrates which led to the development of the mechanism described above were complicated by a fortuitous parallel between substrate hydrophobicity and electron withdrawal.[15] Thus, the contrasting kinetic properties of acetyltyrosine *p*-chloroanilide and acetyltyrosine *p*-methoxyanilide[2b] are attributed[15] to differences in hydrophobicity rather than basicity in the aniline residue. The aniline moiety is presumed to be competed for by two sites on the enzyme (Figure 1); occupancy in the leaving group's site gives a productive complex (I), while occupancy in the hydrophobic site, which ordinarily accommodates the amino acid side chain, gives rise to a nonproductive complex (II). It is proposed that binding mode II predominates with the hydrophobic chloroanilide, and mode I is the main contributor for binding of the less hydrophobic methoxyanilide. This would account for the higher V_{max} of acetyltyrosine *p*-methoxyanilide.[2a,b] Also, since the chloroanilide binds strongly in mode II, and weakly in mode I, while the methoxyanilide only binds relatively weakly in mode I, the apparent binding of the chloroanilide is expected to be tighter. To account for the unusual pH dependence of K_m and V_{max} of the chloroanilide it is further suggested[15] that the nonproductive binding places the hydrophobic amino acid side chain adjacent to Histidine-57, and this is disfavored when the histidine residue is protonated. The low apparent p*K* for both K_m and V_{max} seen with the chloroanilide results.

Taken as support of the proposal that hydrophobicity in the leaving group is of overriding importance in determining substrate affinity is the excellent linear correlation observed between $-\log K_1$ with substituted formanilides and π, the Hansch hydrophobicity constant.[15] The analysis is, however, complicated by the fact that the enzyme has more than one hydrophobic binding site. The preference for hydrophobic groups to bind in the amino acid side chain site is well known. However, the 20-fold higher V_{max}/K_m of Ac-Phe-Ala-NH_2 as compared with Ac-Phe-Gly-NH_2[16] indicates the existence of an additional hydrophobic binding site for the leaving group. This is also suggested from studies of the reaction of nucleophiles with an acyl enzyme[17,18] and studies with substrate analogues.[19] The two sites are

apparently not equally sensitive to hydrophobicity[20] so that the binding of formanilides could be restricted to the mode in which the aniline moiety is in the amino acid side-chain site[22] (nonproductive binding) rather than the leaving group site (productive binding). Although there is no conclusive evidence on this point, it should be noted that the preferred binding mode for the small formanilide substrates provides no information about the binding mode of the larger anilide substrates.

The question of the significance of nonproductive binding of anilide substrates may be treated quantitatively. Calculation of the equilibrium constant for forming each of the 24 possible binding modes of acetyl-D- and L-tyrosine *p*-chloroanilide may be accomplished using a determinant composed of the appropriate microscopic binding factors.[11] The microscopic binding factors for placing the *p*-chloroanilide moiety in the acylamino and α-proton binding sites were assumed to be equal to those previously derived for binding the hydroxybenzyl function.[11b] The two remaining unknowns, the microscopic binding factors for placing this group in the amino acid side-chain site and leaving-group site were calculated from the observed binding, corrected for the presence of organic solvent,[15] of the D and L isomers of acetyltyrosine *p*-chloroanilide. These are equal to 538 and 16.7, respectively. Using the microscopic binding factors it is calculated that with the L substrate the relative occupancy is 28:1 for binding productively (I in Figure 1) and nonproductively (II). This ratio is 0.33 (III):1 (IV) with the D isomer. The so-calculated predominance of productive binding of acetyl-L-tyrosine *p*-chloroanilide rules out the earlier suggestion for accounting for the tight binding and low V_{max} seen with this compound.[15] Also, since the D isomer is calculated to predominantly bind nonproductively (IV) and the observed binding is not significantly pH dependent, it may be concluded that the nonproductive binding modes (II and IV) are not pH-dependent, as previously suggested.[15] The fact that the K_m for acetyltyrosine *p*-acetylanilide is pH dependent[23] although the aniline moiety is not hydrophobic is similarly inconsistent with the notion that the pK perturbation is caused by hydrophobicity-dependent wrong way binding.[15]

A qualitative treatment of the results gives a similar conclusion. If nonproductive binding mode II, in which every group on the α-carbon atom is misplaced, is the predominant mode for binding the L isomer, it is expected that binding of the D derivative will be tighter since the reversal in the placement of the amino acid side chain and leaving group does not misalign the acylamino group and α proton (compare II and IV). The results (Table I) are not in accord with this.

In order for the proposed nonproductive binding mode (II) to be predominant, as has been suggested,[15] the microscopic binding factor for placing the *p*-chloroaniline group in the amino acid side-chain site would have to be equal to 19730. This value is unreasonably high as indicated by comparison with the values calculated[11a] for the *p*-hydroxybenzyl moiety (63.3) or indolylmethyl moiety (340.2). The hydrophobicity of the *p*-chloroaniline group does not appear to be able to provide sufficient driving force to make nonproductive binding important.

The suggestion that variations in the pK influencing V_{max} and K_m with anilide[24] and aliphatic amides[12] results from an interaction between the leaving group and the Histidine-57 charge relay system remains unproven. It is not clear as to what type of interaction might be provided by an aniline moiety for this effect. The failure to observe pK perturbations with nonsubstrate analogs (Table I) would indicate very special steric requirements for the presumed interaction.

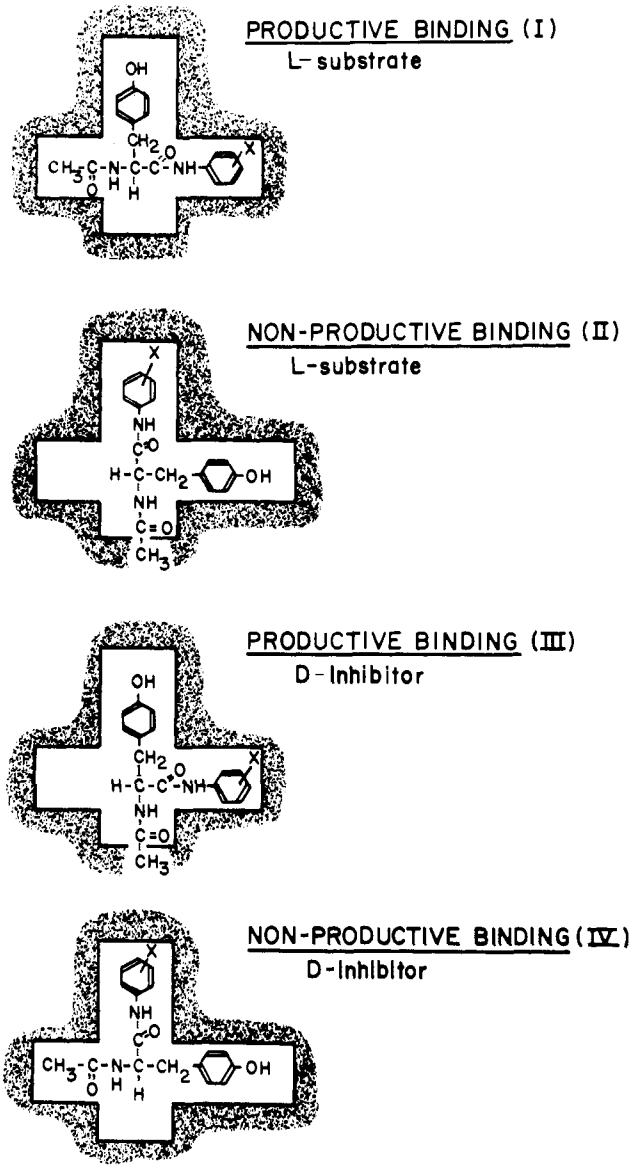
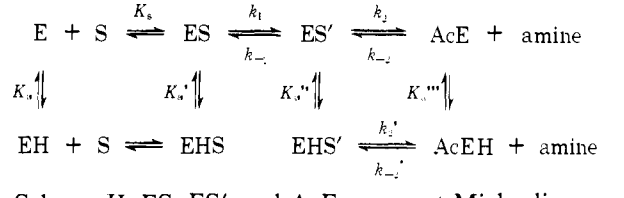


Figure 1. Hypothetical productive and nonproductive binding of acetyltyrosine anilides.

A pH-Induced Change in Rate-Determining Step. The unusually low pK observed for V_{max} with HCO-Phe-FH and the discrepancy seen in the pK influencing V_{max} and K_m (6.08 and 6.7, respectively) has been taken as evidence for a pH-induced change in rate-determining step[2c] (Scheme II).

Scheme II



In Scheme II, ES, ES′, and AcE represent Michaelis complex, tetrahedral intermediate, and acyl enzyme, respectively. At low pH k_2' (EHS′) > k_{-1} (ES′) so that the attack step (k_1) is rate limiting, and at high pH k_{-1} (ES′) > k_2 (ES′) + k_2' (EHS′), so that tetrahedral intermediate breakdown to the acyl enzyme is rate limiting. V_{max} for Scheme II is influenced by two pK's (eq 3). In order to account for

$$V_{max} = \frac{k_1 K_a'[H + (k_2 K_a''/k_2')]}{\{H + [(k_{-1} + k_2)(K_a''/k_2')]\}\{H + K_a'\}} \quad (3)$$

the simple sigmoidal curve reported,[1c] either one of the two pK's must have cancelled. For this to occur and still have a change in rate-limiting step the assignment $k_2/k_2' = K_a'/K_a''$ is required. Under these conditions eq 4 holds and K_a

$$V_{max} = \frac{k_1 K_a'}{H + (k_{-1}K_a'/k_2)} \quad (4)$$

(apparent) for V_{max} is $(k_{-1}K_a')/k_2$. K_m is influenced by a single ionization (eq 5) equal to K_a' in Scheme II, unless K_a

$$K_m = \frac{K_s(K_a')(H + K_a)}{(K_a)(H + K_a')} \quad (5)$$

$= K_a'$, so that K_m is pH independent. Different pK's are, therefore, *required* for V_{max} and K_m in Scheme II.[25]

It was later shown,[2d] however, that the kinetic results did not fit theoretical ionization curves of the assumed pK's of 6.08 and 6.7, and in a repetition of these experiments pK's equal to 6.0 and 6.1 were found for V_{max} and K_m, respectively.

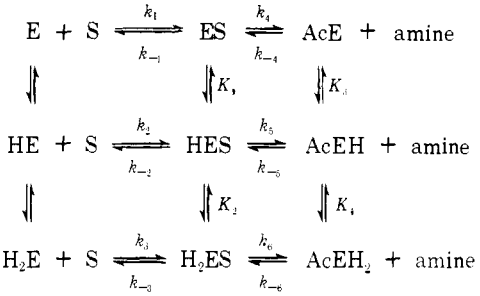
The determination of pK's from kinetic data is subject to considerable error and ambiguity; therefore, we have searched for a change in rate-determining step more directly by studying the microscopic reverse of enzyme acylation, the aminolysis of the acyl enzyme. The distinctive feature of the proposed mechanism is the involvement of both imidazole and imidazolium paths in the formation and breakdown of the tetrahedral intermediate from the acyl enzyme, the k_2, k_{-2}, and k_2', k_{-2}' reactions in Scheme II. To test this mechanism we have looked for the imidazolium path (k_{-2}') in the attack of formylhydrazine on formylphenylalanylchymotrypsin and of *p*-chloroaniline on acetyltyrosylchymotrypsin. We find that there is no reaction involving the imidazole conjugate acid as indicated by the fact that the product ratio is independent of pH (Table III). For Scheme II to hold the ratio of the partitioning of the acyl enzyme between amine and water (amide yield/carboxylic acid yield) at high and low pH is equal to k_2/k_{-1}.[8b] Since $k_{-1} > k_2$ for the proposed mechanism[2c] this ratio is required to be much less than unity. Therefore, the pH independence of the partitioning shows that the proposed pH-induced change in rate-determining step in the acylation of chymotrypsin with HCO-Phe-FH or with Ac-Tyr-NHPhCl does not occur. The results do not exclude a pH-induced change in rate-determining step, if tetrahedral intermediate formation is *exergonic*.

The results given here are most easily understood by considering the fact that in a reaction proceeding by multiple paths (k_2 and k_2' in Scheme II), under identical conditions the same fraction of the reaction that occurs in the forward direction through one path (k_2 as compared with k_2') must proceed through that path in the reverse direction (k_{-2} as compared with k_{-2}'). This is also true at constant pH even if the forward and reverse reactions are run under different conditions, if the transition states for the various paths differ only in the number of protons present. We find, however, that there is no k_{-2}' path under conditions where the k_2' path is presumed to be the predominant element in the acylation reaction. This indicates that there is no k_2' pathway contributing in the acylation reaction.

It was recently reported[12] that the partitioning of an acetylphenylalanylchymotrypsin intermediate between water and formylhydrazine shows what appears to be an inverted bell-shaped pH dependence, with a minimum amide yield of 43.8% at pH 9.8, and 52.4 and 49.5% yields at pH's 5.1 and 10.7, respectively. This result was taken as evidence for the existence of an intermediate and a partial pH-induced change in rate-limiting step. The results cannot be accounted for in terms of Scheme II.

Multiple pH-induced changes in rate-determing step have been suggested for chymotrypsin hydrolysis.[3] The basis of this is the observation that the ^{15}N isotope effect for the hydrolysis of *N*-acetyl-L-tryptophan amide varies with pH. Since the isotope effect is a measure of the extent to which carbon–nitrogen bond cleavage is rate limiting, the changes in the isotope effect indicate a change in the partitioning of the tetrahedral intermediate. In a simplified version containing all of the critical elements of the proposed scheme (Scheme III) the partitioning is governed by k_4/k_{-1} at high pH and k_5/k_{-2} at somewhat lower pH; these ratios must be assumed to be different.

Scheme III



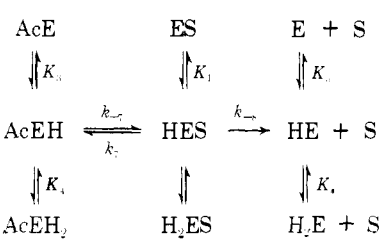
Such a result is surprising since V_{max} for the steady-state hydrolysis of this substrate is pH invariant[26] in the pH range where there presumably is a change in rate-determining step. This requires that $k_4 = k_5$;[27] two different ionic forms of the enzyme have identical catalytic reactivity.

The proposed mechanism (Scheme III) must also describe the reverse reaction, the ammonolysis of an acetyltryptophanylchymotrypsin intermediate. Equation 6 gives

$$P = \frac{\text{yield of amide}}{\text{yield of acid}} = \frac{(NH_3)[k_{-1} + (k_{-2}H/K_t) + (k_{-3}H^2/K_tK_2)] \times [k_{-4} + (k_{-5}H/K_3) + k_{-6}H^2/K_3K_4]K_3(k_7 + k_{-8})}{k_{-8}k_{-7}(H)[k_{-1} + k_4 + (H/K_1) \times (k_{-2} + k_5) + (H^2/K_1K_2)(k_{-3} + k_6)]} \quad (6)$$

the ratio of the products expected to be formed by the competing reaction of water and ammonia with the acyl enzyme. Hydrolysis of the acyl enzyme (Scheme IV) is presumed to not involve multiple paths.

Scheme IV



In general, the product ratio, P, is expected to be pH dependent. For example, at very high pH

$$P = \frac{k_{-1}k_{-4}K_3(k_7 + k_{-8})NH_3}{k_{-7}k_{-8}(k_{-1} + k_4)(H)} \quad (7)$$

and at somewhat lower pH

$$P = \frac{K_3(k_7 + k_{-8})[(k_{-2}k_{-4}/K_1) + (k_{-1}k_{-5})/K_3]NH_3}{k_{-7}k_{-8}(k_{-1} + k_4)} \quad (8)$$

There are a number of other pH regions, each with characteristic partitioning. To test the proposed mechanism it is necessary to study the pH dependence for partitioning (P)

over a sufficient pH range. This is difficult with ammonia. With other amines we find that partitioning with formylhydrazine is independent of pH from pH 7.5 to 4.5 (Table III); partitioning with *p*-chloroaniline is pH invariant from pH 7.5 to 4.5 (Table III); partitioning with ammonia is independent of pH from pH 9.2 to 10.0.[28] Unless it is assumed that the proposed mechanism (Scheme III) is only applicable to the displacement and attack of ammonia, and not valid for the reaction of other amines, these results require the unlikely possibility that eq 6 reduces to a pH-independent form in order that the proposed mechanism (Scheme III) not be ruled out.

Experimental Section

Materials. *N*-Formyl-L-phenylalanine methyl ester was prepared by formylation of L-phenylalanine methyl ester,[29] which had been formed from the methyl ester hydrochloride by treatment with excess triethylamine. After removal of solvent the residual oil was suspended in water and the pH adjusted to 7 with base. The water was evaporated and the product extracted with anhydrous ethyl ether. After removal of the ether a colorless oil was obtained by distillation at 68° and 350 μ using a sublimation apparatus. Anal. Calcd for $C_{11}H_{13}NO_3$: C, 63.76; H, 6.32; N, 6.76. Found: C, 63.60; H, 6.40; N, 6.71.

N-Formyl-D-phenylalanine methyl ester used for subsequent synthesis was prepared in a similar way with omission of the distillation step.

N-Formyl-L-phenylalanine amide was prepared by gently warming 2.0 g of *N*-formyl-L-phenylalanine methyl ester in 50 ml of concentrated aqueous ammonia solution for 10 min. The solution was taken to a low volume under vacuum and the product crystallized from water: mp 167–167.5°. Enzymic hydrolysis indicated near 100% optical purity. Anal. Calcd for $C_{10}H_{12}N_2O_2$: C, 62.49; H, 6.29; N, 14.57. Found: C, 62.69; H, 6.11; N, 14.51.

N-Formyl-D-phenylalanine amide was prepared in an analogous manner: mp 167–168°.

N-Formyl-[U-^{14}C]-L-phenylalanine methyl ester was prepared in a manner similar to the unlabeled material. It had a specific activity of 2.18×10^5 cpm/μmol and 100% purity was indicated by enzymic hydrolysis on a pH stat.

N-[*Carboxyl*-^{14}C]acetyl-L-tyrosine methyl ester was prepared as described previously.[28]

N-Formyl-L-phenylalanine formylhydrazide,[2c] *N*-formyl-D-phenylalanine formylhydrazide, *N*-acetyl-L-tyrosine *p*-chloroanilide,[2b] and *N*-acetyl-D-tyrosine *p*-chloroanilide were prepared as described previously for the L materials.

N-Acetyl-*N*-methyl-L-tyrosine *p*-chloroanilide was synthesized by adding 0.43 g of dicyclohexylcarbodiimide to an ice-chilled mixture of 0.5 g of *N*-acetyl-*N*-methyl-L-tyrosine and 1 g of *p*-chloroaniline in dimethylformamide under nitrogen. After formation of a precipitate the solution was filtered and the filtrate evaporated to dryness. The residual oil was dissolved in ethyl acetate and the color removed by washing with 1 *N* hydrochloric acid. After washing with saturated sodium bicarbonate and water and drying over magnesium sulfate, the product was crystallized from ethyl acetate–petroleum ether: mp 202–203° dec. Anal. Calcd for $C_{18}H_{19}N_2O_3Cl$: C, 62.34; H, 5.48; N, 8.08. Found: C, 62.36; H, 5.63; N, 8.05.

N-Acetyl-*N*-methyl-L-tyrosine methyl ester was prepared as described previously.[14]

N-Acetyl-*N*-methyl-L-tyrosine was prepared by alkaline saponification of the methyl ester and crystallized from ethyl acetate–petroleum ether: mp 177–178°.

N-Methyl-L-phenylalanine was prepared as described previously for *N*-methyl-L-tyrosine.[13]

N-Formyl-*N*-methyl-L-phenylalanine was prepared by formylation of *N*-methyl-L-phenylalanine[29] and crystallized from absolute ethanol: mp 184.5–185.5°. The correct neutralization equivalent of 207 was obtained.

N-Formyl-*N*-methyl-L-phenylalanine formylhydrazide was prepared by adding 2.06 g of dicyclohexylcarbodiimide to an ice-chilled mixture of 2.07 g of *N*-formyl-*N*-methyl-L-phenylalanine and 0.60 g of formylhydrazine in tetrahydrofuran under argon. After 4.5 hr at room temperature the precipitate was filtered off and the filtrate evaporated to dryness. The residue was dissolved in 50 ml of water and the insoluble matter filtered off. Extraction with 6 × 50 ml of ethyl acetate was followed by washing the combined ethyl acetate fractions with 3 × 8 ml of water and drying over magnesium sulfate. Dropwise addition of ethyl ether to the solution, after reduction of volume, produced a semisolid material which solidified upon washing with ether and drying under vacuum at room temperature. In an iodine chamber the product exhibited a single spot of R_f 0.6 on silica gel layers in $CHCl_3$–CH_3OH (9:1); this assay was necessary to follow the removal of an impurity of R_f 0.4 during the washing process. The product is sensitive to atmospheric moisture and does not have a well-defined melting point. ^{1}H NMR in D_2O obtained on a Jeolco C 60HL confirmed the presence of two formyl protons (8.2 and 8.7 ppm) and the *N*-methyl protons (singlet at 3.3 ppm). Anal. Calcd for $C_{12}H_{15}N_3O_3$: C, 57.82; H, 6.06; N, 16.86. Found: C, 57.59; H, 6.19; N, 15.81.

p-Nitrophenyl acetylglycinate was prepared by adding 5.5 g of dicyclohexylcarbodiimide to an ice-chilled mixture of 4 g of acetylglycine and *p*-nitrophenol in dimethylformamide. After stirring overnight at room temperature, filtering off the precipitate, and removing the solvent, the residual oil (dissolved in ethyl acetate) was washed successively with pH 9 carbonate buffer, water, and 1 *N* HCl. The solution was dried over magnesium sulfate and evaporated to low volume. The product was crystallized from ethyl acetate–petroleum ether: mp 129.5–130°.

Methods. Rates of acetyltyrosyl- or formylphenylalanylchymotrypsin aminolysis were determined by product analysis using isotope dilution for quantitation. Reactions were run at 25° in 0.1 *M* KCl (with 10% dimethylformamide for chloroaniline aminolysis) and were initiated by adding enzyme to a solution of [^{14}C]methyl ester and amine. The pH was controlled with a pH stat and reactions were followed to completion as indicated by a cessation of proton release. The solution was heated for 2 min in a boiling water bath to stop further reaction, and the appropriate nonradioactive amide dissolved (35 mg for the chloroanilide; 100 mg for the formylhydrazide). The chloroanilide was allowed to crystallize from this solution and was then recrystallized twice from ethyl acetate–ligroine. The formylhydrazide solution was taken to dryness under vacuum and the residue extracted with 5 ml of 90% ethanol and recrystallized five times from 90% ethanol–ethyl ether. The crystals were dried and their identity confirmed by melting point. In control experiments it was shown that the isolated amides were uncontaminated with the alternate radioactive product of the enzymatic reaction, the acylamino acid. The yield of amide was determined by weight and the radioactivity was measured with a liquid scintillation counter using Aquasol counting fluid. A control experiment in which amine was added after completion of the enzymic hydrolysis of the ester was run to determine the extent of cocrystallization of carboxylate with the amide. The background so-determined was negligible. It was established in control experiments that nonenzymatic aminolysis does not contribute to the amide yield.

Binding constants were measured at 25° by determining the degree of competitive inhibition of the hydrolysis of 17–99 μM *p*-nitrophenyl acetylglycinate, methyl hippurate, or HCO-Phe-FH. For methyl hippurate and HCO-Phe-FH the reaction, followed on a pH stat, was initiated by adding enzyme to 5 ml of solution containing substrate and inhibitor. The hydrolysis of *p*-nitrophenyl acetylglycinate was followed spectrophotometrically at 400 mμ above pH 6.8 and at 330 mμ below that pH. The reaction was initiated by the addition of *p*-nitrophenyl acetylglycinate to a solution containing inhibitor and enzyme. The nonenzymic rate for nitrophenyl ester hydrolysis was determined for each set of reaction conditions and usually accounted for <15% of the total rate.

It was calculated or shown that inhibitors underwent negligible enzymic hydrolysis during the fraction of the reaction followed. Rates of ester hydrolysis were followed to approximately 5% completion. This corresponded to an absorbance change of 0.05 at higher pH and of 0.03 at lower pH for the spectrophotometric assays.

α-Chymotrypsin was three-times crystallized material obtained from Worthington.

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References and Notes

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(2) (a) M. Caplow, *J. Am. Chem. Soc.*, **91**, 3639 (1969); (b) E. C. Lucas and M. Caplow, *ibid.*, **94**, 960 (1972); (c) A. R. Fersht and Y. Requena, *ibid.*, **93**, 7079 (1971); (d) E. C. Lucas, M. Caplow, and K. J. Bush, *ibid.*, **95**, 2670 (1973).

(3) M. H. O'Leary and M. D. Kluetz, *J. Am. Chem. Soc.*, **94**, 3585 (1972).

(4) A. R. Fersht and Y. Requena, *J. Mol. Biol.*, **60**, 279 (1971).

(5) Abbreviations used are HCO-Phe-NH_2, *N*-formyl-L-phenylalanine amide; HCO-D-Phe-NH_2, *N*-formyl-D-phenylalanine amide; HCO-Phe-FH, *N*-formyl-L-phenylalanine formylhydrazide; HCO-D-Phe-FH, *N*-formyl-D-phenylalanine formylhydrazide; HCO-MePhe-FH, *N*-formyl-*N*-methyl-L-phenylalanine formylhydrazide; Ac-Tyr-NHPhCl, *N*-acetyl-L-tyrosine *p*-chloroanilide; Ac-D-Tyr-NHPhCl, *N*-acetyl-D-tyrosine *p*-chloroanilide; Ac-MeTyr-NHPhCl, *N*-acetyl-*N*-methyl-L-tyrosine *p*-chloroanilide.

(6) K. R. Hanson, R. Ling, and E. Havir, *Biochem. Biophys. Res. Commun.*, **29**, 194 (1967).

(7) S. A. Bernhard, W. C. Coles, and J. F. Nowell, *J. Am. Chem. Soc.*, **82**, 3043 (1960).

(8) (a) L. W. Cunningham and C. S. Brown, *J. Biol. Chem.*, **221**, 287 (1956); (b) B. Zeeberg, M. Caswell, and M. Caplow, *J. Am. Chem. Soc.*, **95**, 2734 (1973).

(9) Dr. G. L. Schmir has calculated an equilibrium constant equal to 3×10^{-9} for forming a neutral tetrahedral intermediate in the nonenzymatic intramolecular cyclization of 4-hydroxybutyranilide (private communication).

(10) (a) A. Ruhlman, D. Kukla, P. Schwager, K. Bartels, and R. Huber, *J. Mol. Biol.*, **77**, 417 (1973); (b) R. M. Sweet, H. T. Wright, J. Janin, C. H. Chothia, and D. M. Blow, *Biochemistry*, **13**, 4212 (1974).

(11) (a) C. L. Hamilton, C. Niemann, and G. S. Hammond, *Proc. Natl. Acad. Sci. U.S.A.*, **55**, 664 (1966). (b) Niemann's determinant formulation is based on the idea that for multiple mutually exclusive binding modes, the observed association constant is the sum of the individual association constants for each mode. Evaluation of the positive or negative terms of the determinant is equivalent to adding together the individual association constants for each mode, for an L or D substrate, respectively. Each term to be added is expressed as the product of four microconstants. Sixteen microconstants and two binding constants represent two equalities, so that if 16 of these values are known, the other two can be determined. The microconstants for the placement of a *p*-chloroanilline moiety of *N*-acetyltyrosine *p*-chloroanilide in the side-chain and leaving-group subsites are calculated from the following determinant.

	Subsite on enzyme			
	N-Acyl amino	Side chain	Leaving group	α-H
N-Acetyl	3.67	0.727	0.788	0.400
p-Hydroxybenzyl	1.08	63.3	0.649	0.301
p-Chlorocarboxanilide	1.08	*x*	*y*	0.301
α-H	0.826	0.826	0.826	0.826

For example, 0.727 refers to placement of the *N*-acetyl group into the aromatic side-chain site. After expansion by minors and equating the positive and negative terms with the association constants for the L and D enantiomers, two linear simultaneous equations are obtained.

(12) A. R. Fersht and M. Renard, *Biochemistry*, **13**, 1416 (1974).

(13) R. L. Peterson, K. W. Hubele, and C. Niemann, *Biochemistry*, **2**, 942 (1963).

(14) M. Caplow and C. Harper, *J. Am. Chem. Soc.*, **94**, 6508 (1972).

(15) J. Fastrez and A. R. Fersht, *Biochemistry*, **12**, 1067 (1973).

(16) W. K. Baumann, S. A. Bizzozero, and H. Dutler, *FEBS Lett.*, **8**, 257 (1970).

(17) P. W. Inward and W. P. Jencks, *J. Biol. Chem.*, **240**, 1986 (1965).

(18) A. R. Fersht, D. M. Blow, and J. Fastrez, *Biochemistry*, **12**, 2035 (1973).

(19) C. Hansch and E. Coats, *J. Pharm. Sci.*, **59**, 731 (1970).

(20) A plot of $-\log k$ for the reaction of amines and alcohols with furoylchymotrypsin[17] against the Hansch π value has a slope of 0.43; a plot of $-\log V_{max}/K_m$ for reaction of substituted acetylamino acid methyl esters[21] against π has a slope of 1.8.

(21) J. B. Jones, T. Kunitake, C. Niemann, and G. E. Hein, *J. Am. Chem. Soc.*, **87**, 1777 (1965).

(22) With an enzyme with two hydrophobic sites the dependence of the log of the binding constant on π will be biphasic unless nonhydrophobic binding forces greatly favor binding at one or another of the two sites.

(23) Unpublished result from this laboratory.

(24) (a) M. Philipp, R. M. Pollack, and M. L. Bender, *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 517 (1973); (b) R. Henderson and J. H. Wang, *Annu. Rev. Biophys. Bioeng.*, **1**, 1 (1972).

(25) It was mistakenly reported[2d] that identical p*K*'s for V_{max} and K_m are compatible with Scheme II.

(26) G. Hess, "The Enzymes", 3rd ed, Vol. III, Wiley, New York, N.Y., 1971, p 213.

(27) This is strictly true for the simplified Scheme III. However, in an expanded formulation with more intermediates k_4 and k_5 are not equal to V_{max} but rather V_{max} divided by the concentration of the appropriate intermediate.

(28) B. Zeeberg and M. Caplow, *J. Biol. Chem.*, **248**, 5887 (1973).

(29) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids", Wiley, New York, N.Y., 1961, p 921.

Reactions of Osmium Ligand Complexes with Nucleosides

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Abstract: We have synthesized bis(pyridine)oxoosmium(VI) and 2,2′-bipyridyloxoosmium(VI) esters of the common nucleosides in which osmium is bonded through the 2′- and 3′-hydroxyl groups of the sugar residue. We have also prepared the 2,2′-bipyridyloxoosmium(VI) esters of uridine, cytidine, and thymidine which result from addition of OsO_4 to the 5,6 double bond of the pyrimidine ring. Kinetic studies of the formation of the sugar esters from the nucleoside and the Os(VI) dimer, $Os_2O_6py_4$, give the apparent rate law, $v = k[S][Os(VI)][py]^{-1}[OH^-]^{0.6-0.8}$, in which the hydroxyl ion term reflects hydroxyl ion promoted dissociation of the Os(VI) dimer to monomeric species. The true rate law probably involves three terms, one zero-order, one half-order, and one first-order in hydroxyl ion. The bis(pyridine) esters undergo relatively rapid transesterification reactions with free glycols whereas the 2,2′-bipyridyl esters are much more inert. Kinetic studies of the transesterification reactions give the rate law, $v = k[\text{osmate ester}][\text{glycol}][OH^-][py]^{-1}$.

Osmium derivatives of tRNA have of late assumed considerable importance in the x-ray crystallographic analysis of these species.[1-3] A second application, less developed in a practical way but of considerable potential, is the direct determination of the sequence of bases in nucleic acids by visualization of single heavy atoms.[4-7] In addition to these specific areas where oxoosmium species have already proven to be of importance, there are other interesting consequences and applications of the interactions of metal ions with nucleic acids such as their use in cancer chemotherapy,[8] alterations of the enzymatic specificity of nucleases,[9] and other areas summarized by Clarke and Taube.[10] It is important for these purposes that the chemistry of the reactions used to introduce osmium species into the polymers be understood. This is particularly true of the factors governing specificity and stability. We have to this end undertaken a detailed study of the reactions of oxoosmium complexes with nucleic acid components.[11-14] We report here studies on the stability and exchange reactions of nucleosides with oxoosmium ligand complexes. Preliminary reports of some