The α-Chymotrypsin-catalyzed Hydrolysis of a Series of Acylated Glycine Methyl Esters. II. Behavior at Low and High Substrate Concentrations*

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Activation by excess substrate has been encountered in the α-chymotrypsin-catalyzed hydrolysis of α-*N*-acetyl- and α-*N*-propionylglycine methyl ester. It has not been observed in the hydrolysis of α-methanesulfonyl-, α-*N*-picolinyl-, α-*N*-nicotinyl-, α-*N*-isonicotinyl-, or α-*N*-tetrahydrofuroylglycine methyl ester where the reaction kinetics are described by the simple Michaelis-Menten equation. It is suggested that activation by excess substrate, in the case of α-*N*-acetyl- and α-*N*-propionylglycine methyl ester is achieved by near-neighbor combination of two substrate molecules with the enzyme to form a ternary enzyme-substrate complex which decomposes to products at a faster rate than the binary enzyme-substrate complex because of improved orientation of one of the substrate molecules in the ternary complex.

$R_1'CONHCH_2CO_2CH_3$, the α-*N*-acylated glycine methyl esters, have been of interest as potential substrates of α-chymotrypsin ever since it was shown that methyl hippurate is hydrolyzed by this enzyme (Huang and Niemann, 1952). The earlier studies (Huang and Niemann, 1952, 1953; Applewhite *et al.*, 1958; Applewhite and Niemann, 1959; Hein and Niemann, 1962) not only revealed the existence of this class of substrates but also showed that they were hydrolyzed at the same site involved in the hydrolysis of the more representative substrates of α-chymotrypsin. The α-*N*-acylated glycine methyl esters are interesting because of the probability that this class of structurally deficient bifunctional substrates combines with the active site of the enzyme in modes that frequently are not accessible to the more conventional trifunctional substrates which contain a side chain (Huang and Niemann, 1952, 1953; Hein and Niemann, 1962).

A systematic study of the α-chymotrypsin-catalyzed hydrolysis of α-*N*-acylated glycine methyl esters was initiated by Wolf and Niemann (1963a,b), who examined twenty-three such derivatives generally at relatively low substrate concentrations. In this study we shall be concerned with the kinetic behavior of many of these derivatives over a much wider range of substrate concentrations. In every case the reactions were conducted in aqueous solutions at 25.0°, *p*H 7.90, and 0.50 M in sodium chloride.

The first member of the series of compounds of the type $R_1'CONHCH_2CO_2CH_3$ is α-*N*-formylglycine methyl ester. At *p*H 7.90 and at substrate concentrations from 5.0 to 30.0 mM the base-catalyzed hydrolysis of this compound is so overwhelming relative to the enzyme-catalyzed hydrolysis that the rate of the latter reaction cannot be evaluated (Wolf and Niemann, 1963b). This result is significant because it implies that demonstrable substrate reactivity in this series is contingent upon the presence of an R_1' group larger than a hydrogen atom. It also provides assurance that the structural specificity of the enzyme is sufficiently restrictive to permit distinction between various kinds of methyl esters.

In contrast to α-*N*-formylglycine methyl ester, the rate of the α-chymotrypsin-catalyzed hydrolysis of α-*N*-acetylglycine methyl ester is sufficiently rapid to permit evaluation of its kinetic parameters.

In a study in which substrate concentrations were varied from 5.0 to 600 mM, Wolf and Niemann (1959) concluded from a graphical analysis of the α-chymotrypsin-catalyzed hydrolysis of α-*N*-acetyglycine methyl ester in terms of a v_0 versus $v_0/[S]_0$ plot (Eadie, 1942) that the reaction was capable of activation by excess substrate. The over-all reaction was represented by equations (1) through (4),

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \quad (1)$$

$$ES \xrightarrow{k_2} E + P \quad (2)$$

$$ES + S \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} ES_2 \quad (3)$$

$$ES_2 \xrightarrow{k_4} ES + P \quad (4)$$

from which the rate equation (5):

$$-d[S]/dt = d[P]/dt = \{(k_2K_{S_2} + k_4[S])[E][S]\}/\{K_{S_1}K_{S_2} + K_{S_2}[S] + [S]^2\} \quad (5)$$

where $K_{S_1} = (k_2 + k_{-1})/k_1$ and $K_{S_2} = (k_4 + k_{-3})/k_3$, was derived. Graphical evaluation of the experimental results led to the following values for the kinetic parameters, K_{S_1} = 10 mM, K_{S_2} = 410 mM, k_2 = 0.008 sec^{-1}, and k_4 = 0.069 sec^{-1}. In a subsequent communication Wolf and Niemann (1963a) numerically evaluated the data obtained from experiments in which the substrate concentrations varied from 5.0 to 30.0 mM in terms of equation (6)

$$-d[S]/dt = d[P]/dt = k_0[E][S]/(K_0 + [S]) \quad (6)$$

and obtained values of K_0 = 30.7 ± 5.9 mM and k_0 = 0.013 ± 0.002 sec^{-1}. The preceding results are disconcerting in that the values of K_0 and K_{S_1} and those of k_0 and k_2 should be identical. The fact that they are not is indicative of the presence of a systematic error in the data obtained at *low* substrate concentrations. The gratuitous conclusion of Trowbridge *et al.* (1963) that Wolf and Niemann (1963a) tacitly renounced the existence of activation of the α-chymotrypsin-catalyzed hydrolysis of α-*N*-acetylglycine methyl ester by excess substrate (Wolf and Niemann, 1959) fails to recognize that the analysis of Wolf and Niemann (1959) was based upon data obtained at substrate concentrations which varied from 5.0 to 600 mM, whereas

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TABLE I

α-CHYMOTRYPSIN-CATALYZED HYROLYSIS OF α-N-ACETYLGLYCINE METHYL ESTER[a]

[S] (mM)	$v \times 10^6$ M min^{-1} v_0 (total)	v_B	v_0 (corr)	v_0 (calcd)[b]
5.0	5.19	0.67	4.52	3.96 ± 0.68
10.0	7.92	1.27	6.65	6.90 ± 1.04
15.0	10.56	1.90	8.65	9.27 ± 1.27
20.0	12.39	2.54	9.85	11.3 ± 1.4
25.0	15.40	3.18	12.2	13.1 ± 1.6
30.0	17.09	3.81	13.3	14.8 ± 1.7
50.0	23.71	6.35	17.4	20.6 ± 2.2
100.0	41.09	12.7	28.4	32.1 ± 3.1
150.0	57.64	19.0	38.6	41.6 ± 3.9
200.0	73.02	25.4	47.6	49.8 ± 4.5
250.0	87.43	31.8	55.6	57.2 ± 5.0
300.0	100.8	38.2	62.6	63.3 ± 5.4
500.0	143.1	59.9	83.2	83.0 ± 6.0
600.0	164.7	69.2	95.5	90.5 ± 7.2

[a] In aqueous solutions at 25.0°, pH 7.90, and 0.50 M in sodium chloride with [E] = 4.15×10^{-5} M based upon an assumed molecular weight of 25,000 and a nitrogen content of 16.5% for α-chymotrypsin. [b] Calculated from equation (5) with K_{S_1} = 16.1 ± 2.1 mM, K_{S_2} = 654 ± 60 mM, k_2 = 0.0062 ± 0.0005 sec^{-1}, and k_4 = 0.0701 ± 0.0048 sec^{-1}.

that of Wolf and Niemann (1963a) was restricted to data obtained at substrate concentrations which varied from 5.0 to 30.0 mM.

Numerical analysis (Abrash *et al.*, 1960) is preferable to graphical analysis for the reliable evaluation of data of the kind under consideration. Accepting equation (5) as the rate equation applicable over all ranges of substrate concentration, the experimental observations of Wolf and Niemann (1959, 1963a) have been re-evaluated using numerical procedures designed to achieve the best fit of equation (5) to the experimental data through selection of appropriate values for the kinetic parameters K_{S_1}, K_{S_2}, k_2, and k_4. As will be seen from the data summarized in Table I, reasonable agreement between the experimentally derived and calculated initial velocities is obtained when K_{S_1} = 16.1 ± 2.1 mM, K_{S_2} = 654 ± 60 mM, k_2 = 0.0062 ± 0.0005 sec^{-1}, and k_4 = 0.0701 ± 0.0048 sec^{-1}.

It is now evident that the discrepancy between the values of K_{S_1} and k_2 given above and the values of K_0 = 30.7 ± 5.9 mM and k_0 = 0.013 ± 0.002 sec^{-1}, obtained by a least squares fit of the initial velocities obtained at substrate concentrations which varied between 5.0 and 30.0 mM to equation (6) (Wolf and Niemann, 1963a), arises from a significant participation of the ES_2 reaction at the lower substrate concentrations largely because of the relatively high value of k_4. Therefore, for the ES reaction ideally described by equations (1), (2), and (6), when $K_0 = K_{S_1}$ and $k_0 = k_2$, the values of K_0 = 16.1 ± 2.1 mM and k_0 = 0.0062 ± 0.0005 sec^{-1} are more appropriate than those of K_0 = 30.7 ± 5.9 mM and k_0 = 0.013 ± 0.002 sec^{-1} given by Wolf and Niemann (1963a).

The demonstration that the α-chymotrypsin-catalyzed hydrolysis of α-N-acetylglycine methyl ester, in aqueous solutions at 25.0°, pH 7.90, and 0.50 M in sodium chloride, is activated by excess substrate raises the question as to the generality of this phenomenon. Wolf and Niemann (1963a) observed that the kinetics of the α-chymotrypsin-catalyzed hydrolysis of α-methanesulfonylglycine methyl ester, under the same conditions, are described by equation (6) when the substrate concentrations are varied from 5.2 to 311.6 mM. Thus with the latter substrate there is no indication of activation, or inhibition, of the reaction by excess substrate. From these and the preceding results it is apparent that activation of the reaction by excess substrate is dependent upon the structure of the substrate.

TABLE II

α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF α-N-PROPIONYLGLYCINE METHYL ESTER[a]

[S] (mM)	$v \times 10^6$ M min^{-1} v_0 (total)	v_B[b]	v_0 (corr)	v_0 (calcd)[c]
5.0	5.59	0.78	4.81	5.49
10.0	8.60	1.56	7.04	7.00
15.0	11.8	2.36	9.44	9.59
20.0	14.8	3.12	11.7	11.9
25.0	17.1	3.90	13.2	13.9
30.0	20.0	4.68	15.3	16.0
50.0	29.1	7.80	21.3	22.1
100.0	49.5	15.6	33.9	35.5
150.0	67.2	23.4	43.8	44.0
200.0	82.4	31.2	51.2	50.1
250.0	94.0	39.0	55.0	55.0
300.0	105.7	46.8	58.9	58.7

[a] In aqueous solutions at 25.0°, pH 7.90, and 0.50 M in sodium chloride with [E] = 4.04×10^{-5} M based upon an assumed molecular weight of 25,000 and a nitrogen content of 16.5% for α-chymotrypsin. [b] k_B = 156 M^{-1} min^{-1}. [c] Calculated from equation (5) with K_{S_1} = 9.0 mM, K_{S_2} = 180 mM, k_2 = 0.0033 sec^{-1}, and k_4 = 0.037 sec^{-1}.

The next higher homolog of α-N-acetylglycine methyl ester is α-N-propionylglycine methyl ester. Wolf and Niemann (1963a) examined this compound over a range of substrate concentrations which varied from 5.0 to 30.0 mM and described its reactivity as a substrate of α-chymotrypsin, under the conditions used previously for other members of this series, in terms of equation (6) with values of K_0 = 38.0 ± 6.2 mM and k_0 = 0.018 ± 0.003 sec^{-1}. Examination of the kinetics of hydrolysis of this substrate under the same conditions but over a range of substrate concentrations which varied from 5.0 to 300 mM now have shown that, as with α-N-acetylglycine methyl ester, the enzymatic hydrolysis of α-N-propionylglycine methyl ester also is activated by excess substrate. Because of the greater perturbation of the ES reaction by the ES_2 reaction in the case of α-N-propionylglycine methyl ester, the numerical analysis used for α-N-acetylglycine methyl ester could not be applied. Therefore, a more subjective graphical procedure, based upon use of a v_0 versus $v_0/[S]_0$ plot, had to be used. Again accepting equation (5) as the rate equation, this graphical analysis led to values of K_{S_1} = 9.0 mM, K_{S_2} = 180 mM, k_2 = 0.0033 sec^{-1}, and k_4 = 0.037 sec^{-1}. The experimentally derived initial velocities and those calculated on the basis of equation (5) and the above values of the four kinetic parameters are compared in Table II. The agreement between the two sets of values is the basis for the conclusion that activation by excess substrate is encountered in the α-chymotrypsin-catalyzed hydrolysis of α-N-propionylglycine methyl ester.

The preceding experiments demonstrate that the values of K_0 = 38.0 ± 6.2 mM and k_0 = 0.018 ± 0.003 sec^{-1} obtained by Wolf and Niemann (1963a) from a least squares fit of data, obtained at substrate concentrations which varied from 5.0 to 30.0 mM to equation (6), are in error because equation (5) rather than equation (6) is the proper rate equation for the systems under consideration. Therefore, for the ES reaction encountered in the α-chymotrypsin-catalyzed hydrolysis of α-N-propionylglycine methyl ester, the values of $K_{S_1} = K_0$ = 9.0 mM and $k_2 = k_0$ = 0.0033 sec^{-1} are

more appropriate than those reported previously (Wolf and Niemann, 1963a).

Almond and Niemann (1960) have shown that activation by excess substrate can be simulated by undercorrection for the non-enzyme-catalyzed hydrolysis of substrate. The base-catalyzed hydrolysis of α-*N*-acetylglycine methyl ester was examined under the same conditions and over the same range of substrate concentrations employed in the enzymatic studies (cf. Table I). In this case the base-catalyzed hydrolysis was first order in both substrate and hydroxyl-ion concentrations when the concentration of the latter species was assumed to be given by the *p*H of the system and the substrate concentrations were less than 200 mM. At higher substrate concentrations the base-catalyzed hydrolysis departs from the linear $v_B = k_B[S][OH^-]$ relationship in a negative sense. From these observations we can be confident that activation of the enzymatic hydrolysis of α-*N*-acetylglycine methyl ester by excess substrate is not an artifact caused by undercorrection for the substrate-blank reaction.

Activation by excess substrate also can be simulated by *over*correction for the enzyme-blank reaction (Almond and Niemann, 1960). However, for substrates of the type under consideration, Wolf and Niemann (1963a) concluded that the enzyme-blank reaction was insignificant and consequently no such correction was applied. This practice is supported by the observation that the kinetics of the enzymatic hydrolysis of α-methanesulfonylglycine methyl ester, and as will be shown later those of other substrates of this type are described by equation (6). Since the same considerations apply to the enzymatic hydrolysis of α-*N*-propionylglycine methyl ester, we conclude that, with both α-*N*-acetyl- and α-*N*-propionylglycine methyl ester, activation by excess substrate is not an artifact.

Wolf and Niemann (1963a) found that the kinetics of the α-chymotrypsin-catalyzed hydrolysis of α-*N*-nicotinylglycine methyl ester, at substrate concentrations from 10.0 to 50.0 mM, was described by equation (6) with values of $K_0 = 31.3 \pm 0.4$ mM and $k_0 = 0.460 \pm 0.005$ sec^{-1}. In a subsequent study in which substrate concentrations were varied from 5.0 to 500 mM, Wolf (1959) reported inhibition of the reaction by excess substrate. However in this investigation it was assumed that the substrate-blank reaction, actually determined for substrate concentrations from 10.0 to 30.0 mM, was first order in substrate concentration over the entire range of concentrations. When this assumption was shown to be invalid in the case of α-*N*-acetylglycine methyl ester the substrate-blank reaction for α-*N*-nicotinylglycine methyl ester was reinvestigated and was found to parallel the behavior of that of the former substrate. When proper correction was made for the substrate-blank reaction it was found that the kinetics of the enzymatic hydrolysis of α-*N*-nicotinylglycine methyl ester, at substrate concentrations from 5.0 to 500 mM, were described by equation (6) with substantially the same kinetic parameters reported by Wolf and Niemann (1963a). Thus the apparent inhibition of the reaction by excess substrate (Wolf, 1959) is an artifact caused by *over*correction for the substrate-blank reaction (Almond and Niemann, 1960). The behavior of α-*N*-nicotinylglycine methyl ester is consistent with that observed for α-*N*-isonicotinylglycine methyl ester where the kinetics of the enzymatic hydrolysis also are described by equation (6) for substrate concentrations from 5.0 to 150 mM.

The conclusion that activation of the enzymatic reaction by excess substrate is dependent upon the structure of the substrate arises when it is recalled that such activation was encountered only with α-*N*-acetyl- and α-*N*-propionylglycine methyl ester. At comparable substrate concentrations the kinetics of hydrolysis of α-methanesulfonyl-, α-*N*-nicotinyl-, and α-*N*-isonicotinylglycine methyl ester are described by equation (6), as are those of α-*N*-picolinylglycine methyl ester ([S] = 3.3–49 mM) and α-*N*-tetrahydrofuroylglycine methyl ester ([S] = 18–143 mM) (Rapp, 1963). There is some evidence that activation by excess substrate also may be encountered in the enzymatic hydrolysis of α-*N*-acetylglycine ethyl ester (R. A. Wallace, 1962, unpublished experiments conducted in these laboratories) and possibly in that of *p*-nitrophenylacetate (Kezdy and Bender, 1962). It is now known that the apparent inhibition of the hydrolysis of α-*N*-acetylglycine ethyl ester by excess substrate (Wolf, 1959) is an artifact caused by *over*correction for the substrate-blank reaction.

In an earlier communication, Wolf and Niemann (1963a) gave a tabular summary of the kinetic behavior of twenty-three α-*N*-acylated glycine methyl esters, when examined in aqueous solutions at 25.0°, *p*H 7.90, and 0.50 M in sodium chloride, in terms of the kinetic parameters k_0 and K_0 of equation (6). The information gained in the present study has led to revision of some of the values reported previously and the revised values are given in Table III. The data given in Table III reflect our present knowledge of the kinetic behavior of twenty-four α-*N*-acylated glycine methyl esters. We are confident that equation (6) is the proper rate equation for the eight derivatives containing an arylacyl group and that the values of their kinetic parameters are substantially correct. Of the remaining compounds, the only one that appears to deserve further attention is α-*N*-trifluoroacetylglycine methyl ester. This substrate should be examined at high substrate concentrations to determine whether activation by excess substrate will be encountered. It is unlikely that this phenomenon is an important factor in determining the kinetics of hydrolysis of any of the other substrates listed in Table III, which have not been examined at high substrate concentrations. With the derivatives containing a branched-chain alkyl-acyl component, the inability of α-*N*-pivalylglycine methyl ester to function as a competitive inhibitor (Wolf and Niemann, 1963a) indicates that combination with the active site is impaired by extensive branching in the α-acylamino component even in formation of an ES complex. The pattern observed with the mono-, di-, and trichloroacetyl derivatives, with α-*N*-trichloroacetylglycine methyl ester functioning not as a substrate but as an effective competitive inhibitor (Wolf and Niemann, 1963a), contains no suggestion of activation by excess substrate at higher substrate concentrations. Of the remaining substrates of this group, the greatest uncertainty exists in the case of α-*N*-carbethoxyglycine methyl ester and α-*N*-ethoxyacetylglycine methyl ester, a situation that cannot be resolved until values for the kinetic parameters can be obtained for the latter substrate. The data given in Table III suggest that the enzymatic hydrolysis of α-*N*-*n*-butyrylglycine methyl ester may be accompanied by activation by excess substrate. However, the values of the kinetic parameters obtained for α-*N*-acetyl- and α-*N*-propionylglycine methyl ester indicate that evaluation of the anticipated four kinetic parameters of equation (5) for α-*N*-*n*-butyrylglycine methyl ester would be extremely difficult because of an expected extensive perturbation of the ES reaction by the ES_2 reaction, compounded by an anticipated low rate for the former reaction.

In earlier communications (Wolf and Niemann, 1963a,b) the behavior of a number of substrates was discussed using α-*N*-acetylglycine methyl ester as a

TABLE III

α-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF A SERIES OF ACYLATED GLYCINE METHYL ESTERS[a]

R_1' of $R_1'CONHCH_2CO_2CH_3$	[S] (mM)	K_0[b] (mM)	k_0[b,c] (sec^{-1})	k_0/K_0 (M^{-1} sec^{-1})
H	5.0– 30.1	Too unreactive to evaluate		
CH_3[d]	5.0–600.0	16.1 ± 2.1	0.0062 ± 0.0005	0.385
C_2H_5[e]	5.0–300.0	9 ± 3[p]	0.003 ± 0.001[p]	0.333
$(CH_3)_2CH$	5.0– 30.0	45.4 ± 4.5	0.021 ± 0.002	0.471
$(CH_3)_3C$	5.2– 25.8	Too unreactive to evaluate		
$(CH_3)_2CHCH_2$	5.1– 30.6	24.0 ± 1.6	0.025 ± 0.002	1.02
$(CH_3)_3CCH_2$[f]	39.8	Too unreactive to evaluate		
$ClCH_2$	5.0– 30.0	68.7 ± 1.0	0.055 ± 0.001	0.803
Cl_2CH	5.0– 30.0	21.8 ± 1.4	0.023 ± 0.001	1.06
Cl_3C[g]	5.1– 25.5	Too unreactive to evaluate		
F_3C	5.0– 30.0	53.1 ± 5.0	0.085 ± 0.005	1.59
$C_2H_5OCH_2$	5.1– 30.8	First-order in [S]		0.159
$C_2H_5SCH_2$	5.0– 30.0	10.4 ± 0.1	0.0054 ± 0.0002	0.519
C_2H_5O	5.3– 31.6	65 ± 12	0.131 ± 0.021	2.01
CH_3SO_2[h]	5.2–311.6	85 ± 10	0.012 ± 0.008	0.140
$C_2H_4(CO)_2$[i]	7.9– 47.5	Too unreactive to evaluate		
$C_6H_5CH_2$	2.5– 15.0	7.85 ± 0.50	0.006 ± 0.001	0.777
C_6H_5[j]		7.55 ± 0.30	0.200 ± 0.003	26.49
p-$H_2NC_6H_4$	2.5– 15.0	8.29 ± 0.09	0.041 ± 0.001	4.89
α-C_5H_4N[k]	3.3– 49.0	10.0 ± 1.0[q]	2.46 ± 0.07[q]	246.0
β-C_5H_4N[l]	5.0–500.0	31.3 ± 0.4	0.460 ± 0.005	14.69
γ-C_5H_4N[m]	5.0–150.0	17.7 ± 0.4	0.159 ± 0.001	8.97
α-C_4H_3O[n]	5.0– 30.0	19.6 ± 0.7	0.969 ± 0.028	49.46
β-C_8H_6N[o]	0.7– 4.13	1.37 ± 0.14	0.056 ± 0.003	40.65

[a] In aqueous solutions at 25.0 ± 0.1°, pH 7.90 ± 0.02, and 0.50 M with respect to sodium chloride with $[E] = 3.85 - 4.17 \times 10^{-5}$ M unless otherwise noted. [b] Kinetic parameters with reference to the rate equation $-d[S]/dt = d[P]/dt = k_0[E][S]/(K_0 + [S])$. [c] Based upon a molecular weight of 25,000 and a nitrogen content of 16.5% for α-chymotrypsin. [d] Reaction activated by excess substrate and described by the equation $-d[S]/dt = d[P]/dt = \{k_2K_{S_2} + k_4[S]\}[E][S]/\{K_{S_1}K_{S_2} + K_{S_2}[S] + [S]^2\}$ with $K_{S_1} = K_0 = 16.1 \pm 2.1$ mM, $K_{S_2} = 654 \pm 60$ mM, $k_2 = k_0 = 0.0062 \pm 0.0005$ sec^{-1}, and $k_4 = 0.0701 \pm 0.0048$ sec^{-1}. [e] As described in *d*, but with $K_{S_1} = K_0 = 9.0$ mM, $K_{S_2} = 180$ mM, $k_2 = k_0 = 0.0033$ sec^{-1}, and $k_4 = 0.037$ sec^{-1}. [f] $[E] = 3.51 \times 10^{-5}$ M. [g] A competitive inhibitor with $K_I = 5.3$ mM. [h] General formula does not apply, compound is $CH_3SO_2NHCH_2CO_2CH_3$. [i] General formula does not apply, compound is succinylglycine methyl ester. [j] Determined by Applewhite *et al.* (1958). [k] α-Pyridyl. [l] β-Pyridyl. [m] γ-Pyridyl. [n] α- or 2-Furyl. [o] β- or 3-Indolyl. [p] Indicated error is a subjective estimate. [q] Determined by Rapp (1963).

reference substrate. With revision of the values of $k_0 = k_2$ and $K_0 = K_{S_1}$ for the reference substrate, it is appropriate to consider whether this revision has resulted in abrogation of any of the earlier conclusions. Fortunately, it is not necessary to describe each case in detail since in every case replacement of the original values by the revised values of k_0 and K_0 for α-*N*-acetylglycine methyl ester leads to the same conclusions arrived at earlier.

The unambiguous demonstration of activation of the α-chymotrypsin-catalyzed hydrolysis of α-*N*-acetyl- and α-*N*-propionylglycine methyl ester by excess substrate raises the question as to how activation is achieved in these particular cases. There are at least two explanations that can be given. In one (Alberty, 1956), it can be assumed that the enzyme contains two combining sites, one of which is catalytically active and the other when occupied by substrate leads to activation or inhibition possibly through allosteric modification of the active site (Monod *et al.*, 1963). In the other, in which one active site is assumed, it can be postulated that the molecular dimensions of the active site are greater than those of the substrate, thus permitting a large number of degrees of freedom, with respect to positioning of the substrate in the ES complex, which are diminished by the presence of a second molecule of substrate at the same site. Description of the reaction kinetics in terms of equation (5) does not distinguish between these two alternatives. However, extrakinetic considerations lead us to favor the second of the above alternatives. The relatively low values of K_{S_1} for α-*N*-acetyl- and α-*N*-propionylglycine methyl ester suggest that these molecules readily combine with the active site but are poorly oriented for subsequent transformation into reaction products, judging from their very low values of k_2. Combination of a second molecule of substrate with the ES complex to give an ES_2 complex in which the two substrate molecules are near neighbors, while energetically more difficult than formation of the ES complex, as suggested by the higher values of K_{S_2}, improves orientation of one of the two substrate molecules for subsequent transformation into reaction products to a point where the rate of decomposition of the ES_2 complex into products (k_4) is an order of magnitude greater than that of the ES complex (k_2). With the requirement that the two substrate molecules be near neighbors in the ES_2 complex, and with a site of limited dimensions, one would expect to encounter a marked dependence of activation by excess substrate upon the structure of the substrate, a feature that has been observed. On the basis of present knowledge it appears that with α-chymotrypsin activation by excess substrate will be encountered only with acylated glycine esters where the acyl and ester components are derived from simple unbranched alkyl carboxylic acids and alcohols.

EXPERIMENTAL

The procedures have been described previously (Wolf and Niemann, 1963a). Pertinent experimental details are given in Tables I to III, inclusive.

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The Effects of Coenzymes and Substrates on the Rate of Zinc Exchange in Horse Liver Alcohol Dehydrogenase*

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The rate of exchange of the two zinc atoms of horse liver–alcohol dehydrogenase has been determined by equilibrium dialysis. The isotopically labeled enzyme was exposed to stable zinc ions, and the displacement of ^{65}Zn was measured. Coenzyme, coenzyme moieties, substrates, or substrate homologs alone did not affect the rates of exchange. However, DPN(H), AMP, and ADP ribose *in combination with* substrates or substrate homologs greatly retard the exchange rates. DPNH paired with hexanamide, isobutyramide, or acetamide, and DPN^+ paired with acetate or hydroxylamine were the most effective couples in this regard. *N*-Methylnicotinamide alone or combined with substrates or substrate homologs was completely ineffective in blocking exchange, supporting the view that this moiety is not mandatory for the formation of the enzyme-coenzyme complex (Li and Vallee, 1963; 1964). Some of the substrate homologs which retard exchange, in conjunction with the coenzymes, lack functional groups for binding to the metal atom. The association constants of zinc-DPN(H) complexes have been measured by the ion-exchange method of Schubert (1956). The constants are lower by orders of magnitudes than those of the respective enzyme-coenzyme complexes. Hence interaction of the coenzymes with other, as yet unidentified groups of the apoenzyme must add significantly to the stability of the enzyme-coenzyme complexes.

Recent studies of the mechanism of action of equine liver–alcohol dehydrogenase [(LADH)$^{65}Zn_2$][1] in our laboratory have focused on the binding of coenzymes and substrates, and on the role of zinc in these interactions (Vallee and Coombs, 1959; Vallee *et al.*, 1959; Ulmer *et al.*, 1961; Li *et al.*, 1963). The unique optical properties of the liver-alcohol dehydrogenase–coenzyme complex have permitted direct and detailed examination of the mechanism of coenzyme binding by a variety of approaches (Boyer and Theorell, 1956; Kaplan, 1960; Ulmer *et al.*, 1961; Li *et al.*, 1962; Li and Vallee, 1963, 1964). In contrast, the interaction of substrates and their homologs, which lack suitable chromophoric groups, has had to be studied indirectly, by virtue of their effects on the kinetics of the enzymatic reaction and on the optical properties of the bound coenzyme at equilibrium (Winer and Theorell, 1960; Theorell and McKinley-McKee, 1961; Ulmer *et al.*, 1961).

We have reported that the two firmly bound zinc atoms at the active centers of liver-alcohol dehydrogenase can be exchanged for zinc-65 by equilibrium dialysis (Druyan and Vallee, 1962). When exposed to stable zinc ions, [(LADH)$^{65}Zn_2$] undergoes isotopic exchange, stable Zn^{2+} displacing $^{65}Zn^{2+}$. The rate of exchange measures the reactivity of the zinc atoms at the active sites of the enzyme.

The stability constant of the zinc-enzyme complex is one of the factors which determines the rate at which Zn^{2+} exchanges with $^{65}Zn^{2+}$. However, the binding of coenzymes, coenzyme moieties, substrates, and substrate homologs to the zinc atom or to sites in its proximity would also be expected to influence the rate of exchange. The present study demonstrates that DPN^+, DPNH, and other specific coenzyme moieties, in combination with substrate and substrate homologs, markedly retard isotopic exchange. The stability constants of the zinc-coenzyme (moiety) complexes have also been described.

Materials and Methods

Crystalline horse liver—alcohol dehydrogenase was obtained from C. F. Boehringer und Soehne, Mannheim, W. Germany, and [(LADH)$^{65}Zn_2$] was prepared as described (Druyan and Vallee, 1962). Protein concentration was measured spectrophotometrically at 280 mμ, using an absorbance of 0.455 mg^{-1} cm^2 (Bonnichsen, 1950). The concentration of DPNH

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[1] Abbreviation used in this work: [(LADH)$^{65}Zn_2$], liver-alcohol dehydrogenase.