On the Specificity of Elastase. Hydrolysis of Peptide *p*-Nitrobenzyl Esters†

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ABSTRACT: Five series of *p*-nitrobenzyl esters of peptides having the general formula $Ala_{n-1}X$-ONbz (where n = 2, 3, and 4 and X = Ala, Gly, Val, Phe, or Leu) were synthesized and subjected to enzymic hydrolysis by porcine elastase. All substrates were split at the ester bond. K_m and k_{cat} values were determined for all but two of the substrates. k_{cat}/K_m values increased strongly with n in all series, indicating a specificity with respect to the X residue: Ala > Leu > Val > Gly > Phe, except for n = 4, where Ala ≈ Leu. Judged from K_m values, strength of binding is strongly size dependent and follows Ala > Val > Leu (when $n > 2$). k_{cat} was hardly dependent on peptide length for X = Ala (60–100 sec^{-1}) and X = Val (10–20 sec^{-1}) but for X = Leu it increased dramatically from 20 sec^{-1} (n = 2) to 213 sec^{-1} (n = 3) and 625 sec^{-1} (n = 4). It is suggested that this phenomenon is caused by strain in the "Michaelis complex"—due to the strong binding of the ONbz group—facilitating acylation of the enzyme. Support for this view is found in the observation that in the hydrolysis of peptide bonds in the pair Ala_3Ala–LysAla/Ala_3Leu–LysAla (at the bond), Ala is favored 50-fold over Leu.

Elastase (EC 3.4.4.7), a pancreatic mammalian protease of the "serine-enzyme" type (Brown *et al.*, 1967; Hartley, 1970) seems to have appreciable specificity, judging by the relatively few splits observed when this enzyme acts on natural polypeptide chains of considerable length. Thus, oxidized insulin B-chain (30 residues) is cleaved at a fast rate only at one bond [Ala(14)–Leu(15)] and insulin A-chain (21 residues) at two bonds [Ala(8)–Ser(9) and Ser(12)–Leu(13)] (Narayanan and Anwar, 1969). Similarly, RNase S peptide (20 residues) is cleaved fast at one bond [Ala(6)–Lys(7)] (Atlas *et al.*, 1970). Work with synthetic peptide substrates showed that the rate of hydrolysis is strongly dependent on chain length (Atlas *et al.*, 1970; Thompson and Blout, 1970) establishing the presence of at least six subsites in the enzyme's active site, *i.e.*, S_1, S_2, S_3, S_4, S_1', and S_2' in our nomenclature (Schechter and Berger, 1967).

Elastase also acts as an esterase, hydrolysis rates again depending strongly on the chain length of the substrate (Gertler and Hofman, 1970; Thompson and Blout, 1970). The object of the present study was to investigate, by means of peptide ester substrates, the side-chain specificity of subsite S_1 (binding the residue whose carbonyl function is attacked). This problem has been studied with benzoylamino acid methyl esters (Kaplan *et al.*, 1970) and with carbobenzyloxyamino acid *p*-nitrophenyl esters (Geneste and Bender, 1969). However, as discussed below, these substrates have a number of limitations. In the present study we were able to obtain more detailed information by using five sets of *p*-nitrobenzyl esters of peptides of varying length, having the general structure Ala_{n-1}-X-O-*p*Nb, where the length of the peptide was n = 2–4 (or 5) and X represents Ala, Gly, Val, Leu or Phe.

Materials and Methods

Enzyme Kinetics. Porcine pancreatic elastase was a gift of Dr. D. M. Shotton, who described its preparation in detail (Shotton, 1970). Enzymic hydrolysis rates were determined at 25° by measuring the alkali uptake in a pH-Stat (Radiometer, Copenhagen, type TTT 11 in conjunction with pH-meter type 26) at pH 8.6, in 0.1 M KCl, under an argon atmosphere. The concentrations of titrant (0.2–0.005 M) and of enzyme (2×10^{-6} to 10^{-8} M) were chosen according to the reaction rates, so that initial slopes of 0.5–10 divisions/min were obtained on the record. Blank values (less than 0.5 division/min) were subtracted. Substrate concentrations were usually based on weight. In the case of hygroscopic compounds concentrations of stock solutions were determined by alkali uptake at total enzymic hydrolysis. In all cases it was checked by paper electrophoresis (pH 1.5) that only esterolysis occurred. Enzyme activity of stock solutions (1 mg/ml, kept at 4° for 2 weeks) were checked periodically against Ala_2Lys-AlaOMe ($\bar{K}_m = 1.4 \times 10^4$; k_{cat} = 48 sec^{-1}); no significant decrease in activity was observed.

Substrates. Peptide *p*-nitrobenzyl esters were synthesized stepwise starting from the appropriate L-amino acid *p*-nitrobenzyl ester hydrobromide and *N*-benzyloxycarbonyl-L-alanine as described previously (Schechter and Berger, 1966), or by using the hydroxysuccinimide ester of carbobenzyloxy-alanine or *tert*-butyloxycarbonylalanine (Anderson *et al.*, 1964). Both methods gave essentially the same results. Yields were 70–100%. The *t*-Boc group was removed by 0.5 N HCl in acetic acid (30 min).

The *p*-nitrobenzyl esters of glycine, L-valine, L-leucine, and L-phenylalanine were prepared analogously to that of L-alanine (Schechter and Berger, 1966). Analytical data, etc., for compounds not previously synthesized are given in Table I (intermediates) and Table II (substrates). In addition, all compounds were checked for purity on thin-layer chromatography (tlc) in two solvent systems (chloroform–methanol, 9:1, v/v; butanol–acetic acid–water, 4:1:4, v/v organic layer).

Results

The kinetic parameters ($\bar{K}_m = 1/K_m$ and k_{cat}) were evaluated from double-reciprocal plots of [E]/v ([E] in M, v in M sec^{-1}) *vs.* 1/[S]. Substrate concentrations, [S] (usually varying by a factor of 10 between the highest and lowest concentration), were chosen around or near the K_m value. Plots were

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TABLE I: Analytical Data of Intermediate Compounds.

Compound	Formula	Mp (°C)	Calcd (%)			Found (%)		
			C	H	N	C	H	N
Z-LeuONbz	$C_{21}H_{24}N_2O_6$ (400.42)	59–60	62.99	6.04	7.0	62.89	6.03	6.96
Boc-AlaLeuONbz	$C_{21}H_{31}N_3O_7$ (438.0)	74–76	57.65	7.14	9.61	57.82	7.16	9.83
Z-Ala_2LeuONbz	$C_{24}H_{36}N_4O_8$ (508.0)	98–100	56.68	7.14	11.02	57.44	7.53	11.32
Z-Ala_3LeuONbz	$C_{30}H_{39}N_5O_9$ (613.7)	162–165	58.71	6.41	11.41	58.44	6.35	11.6
Z-GlyONbz	$C_{17}H_{16}N_2O_6$ (344.3)	104–105	59.30	4.68	8.14			8.11
Z-AlaGlyONbz	$C_{20}H_{21}N_3O_7$ (415.39)	213–214	57.83	5.1	10.12	58.0	5.44	
Z-Ala_2GlyONbz	$C_{23}H_{26}N_4O_8$ (486.47)	162–165	56.78	5.39	11.52	56.95	5.43	
Z-Ala_3GlyONbz	$C_{26}H_{31}N_5O_9$ (557.55)	156–160	56.01	5.6	12.56	56.23	5.55	
Boc-ValONbz	$C_{17}H_{24}N_2O_6$ (352.38)	62–63	57.94	6.87	7.95	58.09	6.75	7.88
BocAlaValONbz	$C_{18}H_{29}N_3O_7$ (393.0)	75–77	53.43	7.38	10.68			
Z-Ala_2ValONbz	$C_{26}H_{32}N_4O_8$ (528.5)	172–174	59.08	6.10	10.6	58.90	5.95	10.85
Boc-Ala_3ValONbz	$C_{26}H_{39}N_5O_9$ (565.61)	210–211	55.21	6.95	12.38	55.38	6.77	12.10
Z-PheONbz	$C_{24}H_{22}N_2O_6$ (434.43)	110–112	66.35	5.10	6.45	66.24	5.01	6.65
Boc-AlaPheONbz	$C_{24}H_{29}N_3O_7$ (471.5)	103–105	61.13	6.20	8.91	61.52	6.39	9.18
Z-Ala_2PheONbz	$C_{30}H_{32}N_4O_8$ (576.6)		62.49	5.59	9.72	62.31	5.71	9.71
Boc-Ala_3PheONbz	$C_{30}H_{39}N_5O_9$ (586.0)	190–193	61.43	6.65	11.94			

TABLE II: Analytical Data of Peptide-Ester Substrates.

Substrate	Formula	Mp (°C)	Calcd (%)			Found (%)		
			C	H	N	C	H	N
HBr-LeuONbz	$C_{13}H_{19}BrN_2O_4$ (347.2)	115–117	44.97	5.52	8.07	44.88	4.50	7.90
HCl-AlaLeuONbz	$C_{16}H_{24}ClN_3O_5$ (373.8)	157–159	51.40	6.47	11.24	51.17	6.37	11.05
HBr-Ala_2LeuONbz	$C_{19}H_{29}BrN_4O_6$ (489.3)	179–182	46.63	5.97	11.45			11.94
HBr-Ala_3LeuONbz (1.5H_2O)	$C_{22}H_{37}BrN_5O_{8.5}$ (587.5)	88–90	44.90	6.3	11.80	44.47	6.14	11.41
HBr-GlyONbz	$C_9H_{11}BrN_2O_2$ (291.1)	196–197	37.13	3.81	9.62	37.39	3.65	9.62
HBr-AlaGlyONbz (1H_2O)	$C_{12}H_{18}BrN_3O_6$ (390.0)	148–152	36.90	4.62	11.08	37.38	4.97	10.78
HBr-Ala_2GlyONbz (2H_2O)	$C_{15}H_{25}BrN_4O_8$ (469.3)		38.40	5.31	11.94	37.50	4.98	12.11
HBr-Ala_3GlyONbz (2.5H_2O)	$C_{18}H_{31}BrN_5O_{9.5}$ (549.34)		39.40	5.65		39.52	5.64	
HCl-ValONbz	$C_{12}H_{17}ClN_2O_4$ (288.7)	147–149	49.92	5.94	9.70	50.15	5.93	9.64
HCl-AlaValONbz	$C_{15}H_{22}ClN_3O_5$ (360.0)	170–171	50.01	6.11	11.66	50.37	6.10	11.67
HBr-Ala_2ValONbz (0.5H_2O)	$C_{18}H_{28}BrN_4O_{6.5}$ (484.0)	195–197	44.60	5.78		44.89	5.78	
HCl-Ala_3ValONbz (1H_2O)	$C_{21}H_{34}ClN_5O_8$ (519.9)	190–193	48.40	6.53	13.45	48.03	6.33	13.85
HBr-PheONbz	$C_{16}H_{17}BrN_2O_4$ (381.2)	202–204	50.41	4.49	7.35	50.62	4.54	7.10
HCl-AlaPheONbz	$C_{19}H_{22}ClN_3O_5$ (407.9)	188–190	55.95	5.44	10.30	55.80	5.51	10.14
HBr-Ala_2PheONbz	$C_{22}H_{27}BrN_4O_6$ (524.0)	71–73						
HCl-Ala_3PheONbz	$C_{25}H_{32}ClN_5O_7$ (550.0)	214–215	54.59	5.86	12.73	54.18	6.02	12.94
HCl-Ala_4OMe	$C_{13}H_{25}ClN_4O_5$ (352.8)	202–203	44.25	7.14	15.88	44.29	7.14	16.08
2HCl-Ala_2LysAlaOMe	$C_{16}H_{33}Cl_2N_5O_5$ (444.9)	214–217	43.14	7.42	15.73	43.69	7.54	14.90

linear and the best fitting straight lines were determined by least squares (SE 2–5%). Results are tabulated in Table III. In the case of AlaGlyONbz only the C value ($\bar{K}_m \times k_{cat}$) is given because $\bar{K}_m$ was too small to be determined. AlaPheONbz had so low a reactivity that only an upper limit for the C value could be estimated. Kinetic data for two hexapeptides, Ala_4LysAla (Atlas *et al.*, 1970) and Ala_3LeuLysAla, are also included in the table. In order to facilitate comparison of the data within the series Ala_{n-1}-X-ONb as well as within the series of equal chain length where amino acid X is varied, the data of Table III are graphically represented on a log scale (Figure 1).

Discussion

The "overall" specificity of a protease has usually been defined with respect to the amino acid contributing the carbonyl function of the susceptible bond (*i.e.*, P_1 in our nomenclature, Schechter and Berger, 1967). Despite the fact that a number of proteases do exhibit multiple site binding of substrates, this definition seems to hold for many enzymes (*e.g.*, trypsin and chymotrypsin) meaning that S_1 is the primary discriminating binding subsite. There are two notable exceptions: papain (Schechter and Berger, 1968) and streptococcal proteinase (Gerwin *et al.*, 1966), where the crucial binding is in S_2. In

TABLE III: Kinetic Parameters for the Ester Hydrolysis of Peptide *p*-Nitrobenzyl Esters.

Substrate	$\bar{K}_m$ (M^{-1})	k_{cat} (sec^{-1})	C (M^{-1} sec^{-1})
Ala*Ala*ONbz	70	61	4,300
AlaAla*Ala*ONbz	6,000	67	400,000
AlaAlaAla*Ala*ONbz	24,000	77	1,850,000
AlaAlaAlaAla*Ala*ONbz	12,000	100	1,200,000
AlaAlaAla*Ala*LysAla	1,700	22	37,000
Ala*Val*ONbz	90	10	900
AlaAla*Val*ONbz	1,450	19	26,900
AlaAlaAla*Val*ONbz	22,000	23	500,000
Ala*Leu*ONbz	90	20	1,800
AlaAla*Leu*ONbz	800	213	170,400
AlaAlaAla*Leu*ONbz	3,300	625	2,060,000
AlaAlaAla*Leu*LysAla	120	7	840
Ala*Gly*ONbz			22
AlaAla*Gly*ONbz	460	19	8,550
AlaAlaAla*Gly*ONbz	5,000	74	585,000
Ala*Phe*ONbz			<2
AlaAla*Phe*ONbz	480	0.4	190
AlaAlaAla*Phe*ONbz	2,400	10.5	24,000

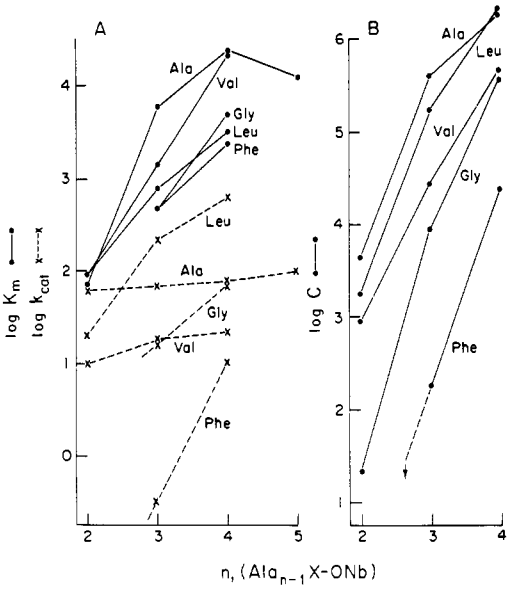

FIGURE 1: The dependence of the kinetic parameters $\bar{K}_m$ ($=1/K_m$), k_{cat}, and C ($=\bar{K}_m k_{cat}$) on the chain length, n, of peptide *p*-nitrobenzyl esters of the general structure Ala$_{n-1}$–X–ONbz. The nature of the X residue (Ala, Val, Gly, Leu, or Phe) for each series is shown in the graph.

carboxypeptidase A, S_1 and S_1' seem to be of comparable importance. Of course, all possible substrate–subsite interactions (up to seven in the case of papain) should contribute to the degree of susceptibility of a given bond in a polypeptide chain (*cf.* carboxypeptidase A; Abramowitz *et al.*, 1967).

It is because of the predominance of the S_1 interaction that small substrates, such as N-blocked amino acid amides and esters, have been used with success in this type of investigation, the latter often having the practical advantages of ease of preparation, higher reactivity, and convenience of assay. The last two points apply especially to *p*-nitrophenyl esters, which are highly reactive (in the acylation step) and give rise to *p*-nitrophenol, which is easily and quickly determined. Evidently, in unknown situations, this practice may be grossly misleading, and a systematic investigation of the various subsites, though very time consuming, may be the only way to get the correct answers.

In the case of elastase, the presence of six subsites has been shown and the influence of binding in S_4 has been pointed out. However, in the work carried out with longer substrates, the bond split was always next to alanine, considered the most "specific" residue on the basis of results obtained with natural polypeptides of known sequence. On the other hand, the S_1 specificity has been investigated by means of N-blocked amino acid esters (Kaplan *et al.*, 1970; Geneste and Bender, 1969) and alanine derivatives were again shown to be the best substrates. The alanine specificity seems to be well supported by evidence of the three-dimensional structure of the active site (Watson *et al.*, 1970) where it can be seen that the pocket binding Phe or Trp in the homologous (Hartley, 1970) chymotrypsin is blocked off by a valine side chain.

In the present study we used the *p*-nitrobenzyl esters of di-, tri-, and tetrapeptides of alanine in which the P_1 residue was systematically substituted by a Gly, Val, Leu, or Phe residue. These substrates have several technical advantages: due to the unblocked amino groups they have good solubilities; they are stable compounds—the reactivity of the ONbz group being comparable to that of the OMe group; they have good binding characteristics, probably due to strong interaction of the *p*-nitrobenzyl group with subsite S_1', making it possible to determine $\bar{K}_m$ and k_{cat} in all but two members of the series investigated. The above is illustrated by comparison to the corresponding methyl esters (Table IV).

Attempting now to interpret the data obtained with the five series of peptides (Table III) in terms of specificity of subsite S_1, let us first compare the $\bar{K}_m \times k_{cat}$ values (Figure 1B), *i.e.*, the pseudo-first-order rate constants at very low substrate concentration. One observes (as in the case of amide-bond cleavage) a strong chain length dependence up to the tetrapeptide, yet within each size group reactivity increases consistently in the order Phe < Gly < Val < Leu < Ala (except that in the tetrapeptide esters Leu "catches up" with Ala). This again confirms the alanine specificity of elastase, but the high reactivity of the Leu bond, especially in the tetrapeptide ester, seems to contradict the findings with the insulin chains (also obtained at concentrations well below K_m (Narayanan and Amwar, 1969)) where no Leu bonds were split (*cf.* Leu(6)–$CySO_3$(7), Leu(11)–Val(12), Leu(15)–Tyr(16), and Leu(17)–Val(18) in B-chain; Leu(13)–Tyr(14) and Leu(16)–Glu(17) in

TABLE IV: Kinetic Parameters of Methyl- and *p*-Nitrobenzyl Esters of Alanine Peptides.

Substrate	$\bar{K}_m$ (M^{-1})	k_{cat} (sec^{-1})	Reference
Ala_3OMe	67	40	Thompson and Blout (1970)
Ala_3ONb	6,000	67	Present work
Ala_4OMe	4,000	50	Present work
Ala_4ONb	24,000	77	Present work

TABLE V: Ratios of Kinetic Constants for the Pairs Ala_3*Leu*-ONbz/Ala_3*Ala*ONb and Ala_3*Leu*LysAla/Ala_3*Ala*LysAla.

	$\bar{K}_m$ (*Leu*)/ $\bar{K}_m$ (*Ala*)	k_{cat} (*Leu*)/ k_{cat} (*Ala*)	*C* (*Leu*)/ *C* (*Ala*)
Ester	0.14	8	1.1
Peptides	0.07	0.3	0.02

A-chain), whereas Val bonds showed distinct though low reactivity [secondary split in B-chain at Val(18)–$CySO_3$(19)], and Gly bonds an even lower one (Gly(23)–Phe(24) on redigestion).

On the other hand, the picture emerging from the breakdown of the *C* values into $\bar{K}_m$ and k_{cat} (Figure 1A) is less straightforward. $\bar{K}_m$ values seem to indicate that binding[1] of the substrates increases in the direction (Phe,Gly,Leu) < Val < Ala with Leu falling behind Val by almost one order of magnitude (in the tetrapeptide ester). The situation is quite different with regard to k_{cat} values. Here the peptides with Ala (60–100 sec^{-1}) and Val (10–20 sec^{-1}) show rather steady values, whereas the k_{cat} of the corresponding leucine peptides increases from 20 sec^{-1} in the dipeptide to over 600 sec^{-1} in the tetrapeptide, by far the highest value observed for any substrate of elastase. In the Gly- and Phe-containing dipeptides $\bar{K}_m$ and k_{cat} values seem to be very low indeed and could therefore not be determined. In the corresponding tri- and tetrapeptides the k_{cat} of the Phe peptides is considerably lower than that of the Gly peptides (almost two orders of magnitude in the former case, almost one in the latter), both increasing steeply with the chain length.

A tentative interpretation of these observations may be the following. First, elongation of the peptides offers additional points of attachment to the enzyme surface (S_3 and S_4) and therefore binding increases in all cases up to a length of four residues. The fact that the alanine peptides are bound considerably better than the corresponding ones containing Gly residues in P_1 indicates a favorable interaction of the additional methyl group in S_1. The same may be said about the isopropyl group of valine. The constancy (with chain length) of the k_{cat} values for Ala and Val peptides indicates that the mode of binding is such that the presence of additional anchoring points in S_3 and S_4 does not change the geometry of the susceptible bond *vis-à-vis* the catalytic system of the enzyme during the acylation process. On the other hand, the consistently lower k_{cat} values of the Val peptides indicate a less favorable alignment caused by the presence of the two extra methyl groups in a tight environment. The differences between Val and Leu peptides seem at first rather surprising, being caused by the presence of the β-methylene which removes the isopropyl group by 1.5 Å from the backbone. However, it may be that in this case part of the extra binding energy from interaction in S_3 and S_4 is absorbed in strain at the ester bond being broken, thus lowering the energy barrier for the formation of the tetrahedral intermediate leading to acylation of the enzyme. That would explain the steep increase with chain length in the k_{cat} values of the Leu peptides. The high absolute value of k_{cat} for Ala_3LeuONbz (625 sec^{-1}) would in itself indicate the presence of a strained intermediate.

The low k_{cat} values for the Phe peptides indicate a generally unfavorable geometry at the catalytic site, the improvement with size being due to either better alignment or the appearance of strain or a combination of both factors. In all the above arguments it should be remembered that the nitrobenzyl ester group of the substrates is strongly bound in S_1'.

In view of the above it is now relevant to ask whether the findings presented here, mainly with respect to the high reactivity of a Leu bond, apply also to "pure" peptide chains, where S_1' and S_2' are occupied by amino acid residues, or whether the strongly bound nitrobenzyl group is responsible for the effects observed.

Experiments are being carried out now with *n*-peptides of the structure Ala_{n-2}-X-LysAla in order to answer this question. Values obtained for Ala_3LeuLysAla (see Table III) in conjunction with the ones published earlier for Ala_4LysAla provide already a clue in that direction. Comparison of the corresponding two pairs (see Table V) shows that in the peptide the Leu bond is indeed only 2% as reactive as the Ala bond (*C* values), and not of equal reactivity as in the esters. The main reason for this is the reversion in the k_{cat} ratios.

Although it is gratifying that this additional bit of information seems to reconcile the discrepancies pointed out above, one is led to the conclusion that "specificity" is often not easily defined and may depend quite strongly on the nature of the system used for its study.

[1] $\bar{K}_m$ seems to us to be close to the reversible association constant K_s ($= k_1/k_{-1}$). The argument runs as follows. From the steady-state treatment of the three-step mechanism (binding (k_1, k_{-1}), acylation (k_2), and deacylation (k_3)), one gets $\bar{K}_m = k_1(k_2 + k_3)/(k_{-1} + k_2)k_3$ or, assuming $k_{-1} \gg k_2$: $\bar{K}_m = K_s[1 + (k_2/k_3)]$. Now, if the substrates dealt with are poor acylators, k_2/k_3 will be about unity or less, and $\bar{K}_m \approx K_s$. That this is indeed the case is supported by the following findings. (a) k_2 seems to be rate limiting in peptide hydrolysis since substrates with the same acyl moiety, *i.e.*, with the same k_3, have different k_{cat} [$= k_2k_3/(k_2 + k_3)$] values: Ala_3LysPhe, 2.5 sec^{-1} (Atlas *et al.*, 1970), *vs.* Ala_3-OMe, 40 sec^{-1}, and Ala_3ONbz, 67 sec^{-1}. (b) Methyl esters are not much better acylators than peptides since $\bar{K}_m$ values are similar: Ala_3LysPhe, 40 M^{-1}, *vs.* Ala_3OMe, 67 M^{-1}. (c) Benzyl esters are chemically not more reactive than methyl esters and therefore the large $\bar{K}_m$ values of Ala_3-ONb, 6000 M^{-1}, as compared to $\bar{K}_m = 67$ M^{-1} for the corresponding methyl ester must stem from its strong binding (large K_s).

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Guanine Phosphoribosyltransferase from *Escherichia coli*, Specificity and Properties†

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ABSTRACT: The specificity and properties of a novel guanine phosphoribosyltransferase of *Escherichia coli* were studied and compared to those of the hypoxanthine–guanine phosphoribosyltransferase from other sources. The structural requirements for binding of purines to this enzyme were explored by the determination of the K_i values for 100 purines and purine analogs. The most effective binding occurred when the purine contained an oxo or thio group in the 6 position and an amino or hydroxyl group in the 2 position. Unlike the hypoxanthine–guanine phosphoribosyltransferase from other sources, this enzyme bound hypoxanthine 67 times less effectively than guanine and four times less effectively than xanthine. Rates of nucleotide formation from a number of purines and purine analogs were also determined. The enzyme had a pH optimum from 7.4 to 8.2. From secondary double-reciprocal plots derived from an initial velocity analysis, the K_m values were 0.037 mM for guanine and 0.33 mM for 5-phosphoribosyl 1-pyrophosphate. The enzyme was sensitive to inhibition by *p*-chloromercuribenzoate and this inhibition could be reversed by either dithiothreitol or β-mercaptoethanol. The apparent activation energy with guanine as the substrate was 12,800 cal/mol below 23° and 3370 cal/mol above 23°. Using isoelectric focusing, the guanine phosphoribosyltransferase had an apparent p*I* of 5.50 while the p*I* of a second enzyme which was specific for hypoxanthine was 4.8.

Phosphoribosyltransferases (PRTases)[1] which catalyze the condensation of purine bases and PP-ribose-P to form 5′-ribonucleotides are widely distributed in nature. Mammals have two such enzymes. One has specificity for 6-aminopurines (adenosine monophosphate:pyrophosphate phosphoribosyltransferase, EC 2.4.2.7) (Henderson and Gadd, 1968; Krenitsky *et al.*, 1969a) and the other for 6-oxopurines (inosine monophosphate:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) (Krenitsky *et al.*, 1969b; Miller and Bieber, 1969). The latter enzyme isolated from a number of different sources has been shown to be the same enzyme which converts with similar efficiency both hypoxanthine and guanine to their respective ribonucleotides (Krenitsky *et al.*, 1969b; Miller and Bieber, 1969; Henderson, 1969). In addition to this pair of enzymes, some microorganisms have yet another PRTase which exhibits specificity for xanthine (Kalle and Gots, 1961; Krenitsky *et al.*, 1970). With *E. coli* a different set of three distinct PRTases has been described (Krenitsky *et al.*, 1970). One, like that of other organisms, is specific for 6-aminopurines. Another shows specificity for hypoxanthine and the third acts preferentially on guanine and xanthine. The specificity and properties of the latter enzyme are the subjects of this report.

Materials

Ampholytes were purchased from LKB. Ultra Pure Tris, sucrose, and [2-^{14}C]uracil were purchased from Schwarz/Mann. 4-Hydroxypyrazolo[3,4-*d*][6-^{14}C]pyrimidine and 4,6-dihydroxypyrazolo[3,4-*d*][6-^{14}C]pyrimidine were synthesized in this laboratory (Elion *et al.*, 1966). 2,6-Diamino[8-^{14}C]purine was a gift from Dr. M. Earl Balis of the Sloan-Kettering Institute, New York, N. Y. 6-Mercapto[8-^{14}C]purine was purchased from New England Nuclear Corp. Ecteola-cellulose (Cellex E) was purchased from Bio-Rad Laboratories. Uric acid was purchased from Doughterty Chemical Co.; 8-chloroxanthine and 9-methylguanine from Cyclo Chemical; 1,3-dimethylxanthine from Mallinckrodt Chemical Works; 2,4-dihydroxy-5-aminopyrimidine from Eastman Kodak Co.; guanine, hypoxanthine, xanthine, adenine, purine, 8-bromoguanine, isocytosine, thymine, uracil, 5-bromouracil, 2-amino-4,6-dihydroxypyrimidine, 2-amino-4,6-dimercaptopyrimidine, and 2,4-dihydroxy-6-methylpyrimidine from Sigma Chemical Co. Synthesized in these laboratories were the following: 1-methyl-6-thiopurine and 6-mercapto-9-methylpurine (Elion, 1962); 2-methylamino-6-hydroxypurine (Montgomery and Holum, 1958); 2-dimethylamino-6-hydroxypurine, 2-anilino-6-hydroxypurine, and 2-methylthio-6-hydroxypurine (Elion *et al.*, 1956a); 8-methylguanine (Traube, 1923); 8-methylxanthine (Fischer *et al.*, 1952); 8-hydroxyguanine (Fischer, 1897); 8-mercaptoguanine and 8-methylthioguanine (Elion *et al.*, 1959); 8-aminoguanine (Jones and Robins, 1960); 8-phenylguanine (Elion *et al.*, 1951); 2-acetylamino-6-mercaptopurine (Serkagaku, Kogyo Co., Ltd., 1966); 2-chloro-6-mercaptopurine, 2,6-dimercaptopurine, and 2-methyl-6-mercaptopurine (Hitchings and Elion, 1954); 2-amino-6-mercapto-7-methylpurine (Prasad and Robins, 1957); 2-amino-6-mercapto-9-methylpurine, 2-amino-6-mercapto-9-ethylpurine, 2-amino-6-mercapto-9-*n*-propylpurine, and 2-amino-6-mercapto-9-*n*-butylpurine (Noell and Robins, 1962); 2-amino-6-chloropurine (Hitchings, 1957); 6-carbethoxypurine (Well-

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[1] Abbreviations used are: PP-ribose-P, 5-phosphoribosyl 1-pyrophosphate; *p*-CMB, *p*-chloromercuribenzoate; PRTase, phosphoribosyltransferase; PRT, phosphoribosyl transfer.