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Nonpolar Effects in Reactions of the Sulfhydryl Group of Papain*

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ABSTRACT: A series of *N*-alkylmaleimides, *N*-ethylmaleimide, and *N*-butylmaleimide to *N*-decylmaleimide, inclusive, were shown to inactivate papain effectively at neutral pH through a pseudo-first-order process.

The apparent second-order rate constants for papain inactivation were demonstrated to increase with increasing chain length of the maleimide derivatives. Titration of the enzyme with *N*-pentylmaleimide indicated that only one functional group of the enzyme reacted with the maleimide and a 1:1 stoichiometry existed between the modification of this functional group and the loss of catalytic activity. Saturation kinetics were obtained in the maleimide inactivation of papain and the absence of chain-length effects in the maximum rate of inactivation with four different *N*-alkylmaleimides indicated that the involvement of alkyl side chains in the overall inactivation process occurs predominantly in the binding of maleimides to the enzyme. The maleimide inactivation of papain was described as a two-step process, the binding of maleimides at the active site which is facilitated by nonpolar interactions of the alkyl side chains of the maleimide derivatives with the nonpolar cleft region of the enzyme followed by an irreversible inactivating reaction between the bound maleimide and the active-site sulfhydryl group. α-*N*-Benzoyl-L-arginine, a substrate-competitive inhibitor of papain, was shown to provide some protection against maleimide inactivation.

The papain-catalyzed hydrolysis of a number of substrates has been reported to proceed through the formation of an acyl-enzyme intermediate. Smith *et al.* (1955) indicated that the acyl-papain intermediate is a thiol ester involving the essential sulfhydryl group of the enzyme. Several more recent studies have contributed evidence in confirmation of this process (Bender and Brubacher, 1964; Lowe and Williams, 1965; Kirsch and Igelstrom 1966). Inactivation of papain by alkylation was attributed to specific modification of the essential cysteine residue of the enzyme (Balls and Lineweaver, 1939; Finkle and Smith, 1958; Hollaway *et al.*, 1964; Light, 1964; Sluyterman 1968). In peptide-sequencing studies, Light *et al.* (1964) demonstrated the active-site sulfhydryl and only sulfhydryl of the enzyme to be Cys-25. X-Ray studies of crystalline papain (Drenth *et al.*, 1968) have revealed that the active-site cysteine residue lies in a nonpolar cleft region of the enzyme. Recent studies of the papain sulfhydryl group (Sluyterman, 1968; Chaiken and Smith, 1969a; Chaiken and Smith, 1969b) have demonstrated that other ionizable groups of the enzyme can influence reactions of the essential sulfhydryl group. The present study provides evidence that the nonpolar cleft region of the enzyme can also affect reactions of the active-site sulfhydryl group.

Experimental Section

Materials. Twice-crystallized papain suspended in 0.05 M sodium acetate, pH 4.5 (19 Bz-L-AgEt units per mg), *N*-carbobenzoxyglycine *p*-nitrophenyl ester, and α-*N*-benzoyl-L-arginine were obtained from the Sigma Chemical Co. Stock solutions of the enzyme were prepared by diluting the crystalline enzyme suspension with 0.02 M potassium phosphate, pH 6.8. Stock solutions of the substrate, *N*-carbobenzoxyglycine *p*-nitrophenyl ester (1.5×10^{-2} M), were prepared in acetonitrile. *N*-Ethylmaleimide was purchased from Eastman Organic Chemicals. *N*-Butylmaleimide was obtained from Nutritional Biochemical Corp. The *N*-pentyl-, *N*-hexyl-, *N*-heptyl-, *N*-octyl-, *N*-nonyl-, *N*-decyl-, and *N*-phenylmaleimides and the *N*-hexyl-, *N*-octyl-, and *N*-phenylmaleamic acids were prepared according to Heitz *et al.* (1968).

Methods. Activation of papain (1.86×10^{-6} M) was carried

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TABLE I: Apparent Second-Order Rate Constants for Maleimide Inactivation of Papain.

Maleimide Derivative	Concentration Range Studied (M)	Apparent Second-Order Rate Constant (M^{-1} min^{-1})
N-Ethyl	1×10^{-4}–6×10^{-4}	166
N-Butyl	4×10^{-5}–2×10^{-4}	260
N-Pentyl	7×10^{-5}–3×10^{-4}	410
N-Hexyl	1×10^{-4}–3×10^{-4}	720
N-Heptyl	4×10^{-5}–8×10^{-5}	3,050
N-Octyl	1×10^{-5}–3×10^{-5}	5,200
N-Nonyl	4×10^{-6}–8×10^{-6}	8,100
N-Decyl	3×10^{-6}–6×10^{-6}	21,450
N-Phenyl	5×10^{-5}–3×10^{-4}	500

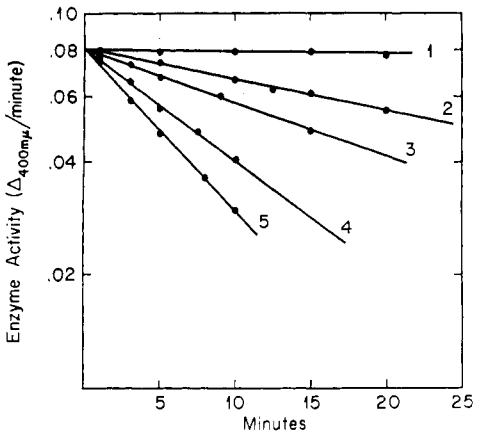

FIGURE 1: Time-dependent inactivation of activated papain by *N*-ethylmaleimide at 25°. The reaction mixtures contained 0.12 M potassium phosphate buffer, pH 6.8, 9.3×10^{-7} M papain, and 0.5% ethanol: line 1, no inhibitor; line 2, 1×10^{-4} M *N*-ethylmaleimide; line 3, 2×10^{-4} M *N*-ethylmaleimide; line 4, 4×10^{-4} M *N*-ethylmaleimide; line 5, 6×10^{-4} M *N*-ethylmaleimide. Catalytic activity was determined as described in the Methods section.

out at 37° in 0.22 M potassium phosphate, pH 6.8, containing 0.05 M potassium cyanide and 7×10^{-3} M EDTA. A similar activation process was used previously by Morihara (1967). Using this procedure, maximum catalytic activity was obtained in 40 min and remained constant for at least 6 hr thereafter. The enzyme was routinely activated for 60 min prior to study.

The catalytic activity of papain was measured spectrophotometrically at 25° using *N*-carbobenzoxyglycine *p*-nitrophenyl ester (Kirsch and Igelstrom, 1966) as substrate. The assay mixtures contained 0.014 M potassium phosphate, pH 6.8, 9.6×10^{-5} M substrate, 9.3×10^{-8} M papain, 0.05% ethanol, and 0.64% acetonitrile in a total volume of 3.12 ml. Reactions were initiated by addition of substrate and hydrolysis was followed at 400 mμ.

Maleimide inactivation studies were carried out at 25° in 2-ml reaction mixtures containing 0.12 M potassium phosphate, pH 6.8, 9.3×10^{-8} M papain, 0.5% ethanol, and the maleimide derivative, the concentration of which will be indicated in individual experiments. Aliquots were removed from these incubation mixtures and assayed for catalytic activity by tenfold dilution into the reaction mixtures described above. Stock solutions of *N*-alkylmaleimides were prepared fresh daily in 2% ethanol.

Spectrophotometric measurements were carried out in a temperature-controlled cell compartment of a Zeiss PMQ II spectrophotometer or a Gilford Model 2000 recording spectrophotometer. Measurements of pH were made at 25° with a Radiometer pH meter, type PHM 4c with a G-200-B glass electrode.

Results

Twice-crystallized papain, activated with potassium cyanide, was incubated at 25° with *N*-alkylmaleimides of varying chain length. Aliquots of the incubation mixtures were removed and assayed under optimal conditions for catalytic activity. Incubation with the various *N*-alkylmaleimides at concentrations in excess of the enzyme concentration resulted in a time-dependent loss of catalytic activity which followed pseudo-first-order kinetics. The inactivation of papain by *N*-ethylmaleimide is characteristic of the inactivation reaction observed with all of the *N*-alkylmaleimides tested and is shown in Figure 1. Incubation of papain under the same conditions, in the absence of *N*-alkylmaleimides (Figure 1, line 1) resulted in no appreciable loss of catalytic activity over a comparable time period. The rate of inactivation of papain by *N*-ethylmaleimide was observed to increase linearly with *N*-ethylmaleimide concentration yielding an apparent second-order rate constant of 166 M^{-1} min^{-1}. In this manner, second-order rate constants for papain inactivation were determined for a series of *N*-alkylmaleimides ranging in size from the *N*-butyl to the *N*-decyl derivative, inclusive. In all cases, when the concentration of the maleimide derivative was held in excess of the enzyme concentration, pseudo-first-order rates of inactivation were observed. The concentration range in which the various *N*-alkylmaleimides were studied and the apparent second-order rate constants for papain inactivation are shown in Table I. The effectiveness of *N*-alkylmaleimides as inactivators of the enzyme increases with increasing chain length of the alkyl substituent of the maleimide derivative. The chain-length effect on apparent second-order rate constants of inactivation is shown in Figure 2.

N-Phenylmaleimide, *N*-phenylmaleamic acid, *N*-hexylmaleamic acid, and *N*-octylmaleamic acid were studied as possible inactivators of papain under conditions identical with those described above for studies of *N*-alkylmaleimides. *N*-Phenylmaleimide was observed to inactivate the enzyme in a pseudo-first-order process and the apparent second-order rate constant for this inactivation is listed in Table I. In concentration ranges equal to those used for the corresponding substituted maleimides, the substituted maleamic acids showed no appreciable inactivation of papain over a 30-min incubation period.

A substrate-competitive inhibitor of papain, α-*N*-benzoyl-L-arginine (Sluyterman, 1964), was studied with respect to the ability to protect the enzyme against maleimide inactivation. It was first necessary to determine the properties of α-*N*-benzoyl-L-arginine inhibition of papain under conditions employed in the present study. Initial velocity measurements were made under assay conditions, varying substrate concen-

TABLE II: Titration of Papain with *N*-Pentylmaleimide.

Enzyme Concentration (M)	Maleimide Concentration (M)	Moles of Enzyme/ Moles of Maleimide	Maleimide Reacted (M)	Enzyme Reacted (Equiv)	% Loss of Catalytic Activity
3.7×10^{-5}	3.7×10^{-5}	1.0	2.96×10^{-5}	0.80	80
1.66×10^{-5}	5×10^{-5}	0.33	1.4×10^{-5}	0.85	89
1.66×10^{-5}	8×10^{-5}	0.21	1.68×10^{-5}	0.99	93
1.66×10^{-5}	8.3×10^{-6}	2.0	8.3×10^{-6}	0.5	50
1.66×10^{-5}	4.15×10^{-6}	4.0	4.15×10^{-6}	0.25	20

tration in the absence and in the presence of two concentrations of α-*N*-benzoyl-L-arginine. These data plotted according to Lineweaver and Burk (1934) indicated that the inhibition by this compound under these conditions was competitive with respect to substrate and an inhibitor dissociation constant (K_I) of 2.5×10^{-3} M was calculated. The rate of inactivation of papain by *N*-pentylmaleimide was then studied in the presence of this competitive inhibitor. The inactivation process was studied as described above using 5×10^{-4} M *N*-pentylmaleimide. In the presence of concentrations of α-*N*-benzoyl-L-arginine equaling 1.2 and 2 times the K_I value, the rate of papain inactivation was reduced by 24 and 50%, respectively.

The extent of the reaction of maleimides with the sulfhydryl group of papain can be determined by measuring the decrease in the 300-mμ absorption of the maleimide derivatives upon reaction (Alexander, 1958). Activated papain was incubated at 25° in 12-ml reaction mixtures containing 0.12 M potassium phosphate, pH 6.8, 0.5% ethanol, and *N*-pentylmaleimide. The change in absorbance at 300 mμ using 5-cm light-path cuvets was followed for 2 hr at 25° and then the reaction mixtures were allowed to stand 18 hr longer at 4° to ensure reaction completion. Maleimide solutions were standardized by reaction with cysteine under the same conditions. The results of the titration of papain with *N*-pentylmaleimide are shown in Table II. It can be seen that the percentage loss of catalytic activity parallels the equivalents of enzyme reacting with the maleimide. When the *N*-pentylmaleimide concentration was approximately five times that of the enzyme, only 0.99 equiv of enzyme reacted and this was accompanied by a 93% loss of catalytic activity.

The observed pseudo-first-order rate constants for maleimide inactivation of papain show a linear relationship with respect to maleimide concentration within a given concentration range; however, if one continues to increase the maleimide concentration beyond this range, one observes a pronounced deviation from linearity, a tendency toward a maximum rate of inactivation. This is exemplified by the rates of inactivation obtained at higher concentrations of *N*-pentylmaleimide (Figure 3). It is for this reason that the slopes of the linear portions of these curves were previously referred to as apparent second-order rate constants. If this deviation from linearity represents a protein binding of the maleimides prior to the inactivating sulfhydryl reaction, then extrapolation to infinite maleimide concentration should yield the maximum rate of inactivation. Plots of the reciprocal of the observed pseudo-first-order rate constants *vs.* the reciprocal of

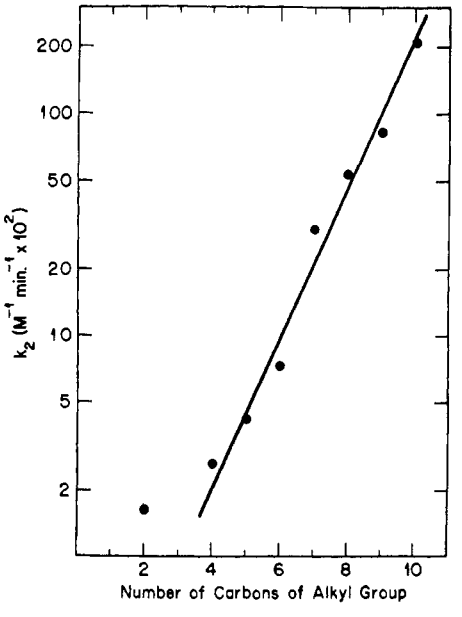

FIGURE 2: The relationship of the logarithm of the apparent second-order rate constants to the chain length of the alkyl substituents of the *N*-alkylmaleimides.

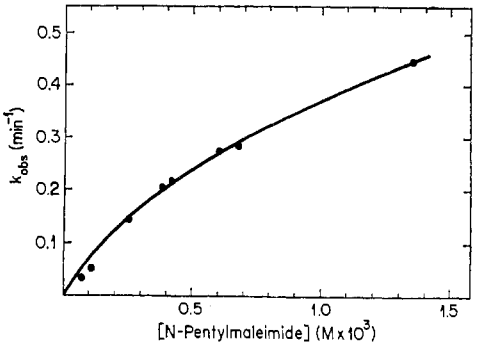

FIGURE 3: The effect of high concentrations of *N*-pentylmaleimide on rates of inactivation of papain. Incubation mixtures contained 0.12 M potassium phosphate buffer, pH 6.8, 9.3×10^{-7} M papain, and 0.5% ethanol.

maleimide concentrations are shown in Figure 4 for *N*-butyl-, *N*-pentyl-, and *N*-hexylmaleimides. Extrapolation in each case yields the same maximum rate of inactivation, 0.5 min^{-1}. Although not shown in this figure, the same maximum rate of inactivation was also obtained with *N*-heptylmaleimide.

Discussion

In the inactive native form of papain the active-site sulfhydryl has been demonstrated to exist in a disulfide linkage with free cysteine (Klein and Kirsch, 1969). Activation by cyanide releases the active-site sulfhydryl converting the once-attached cysteine into β-thiocyanoalanine which cyclizes to 2-iminothiazolidine-4-carboxylic acid. This process provides a means of studying the active-site sulfhydryl group in the absence of other thiol compounds previously used for activation of the enzyme. The catalytic activity of cyanide-activated papain has been demonstrated to be lost on incubation with *N*-alkylmaleimides. This inactivation process followed pseudo-first-order kinetics when studied at maleimide concentrations in excess of the enzyme concentration (Figure 1). Inactivation by maleimides is in itself not a novel contribution to our understanding of the functioning of the papain molecule since inactivation of this enzyme by sulfhydryl reagents has been adequately established in many earlier and excellent studies. In fact, in several of the earlier studies, such as those reported by Sluyterman (1968), it has been pointed out that the papain sulfhydryl group is unusually reactive toward sulfhydryl reagents. However, it is felt that the observation of maleimide inactivation of papain proceeding through a process facilitated by increasing chain-length of the maleimide derivatives (Figure 2) is related to the nature of the immediate environment of this sulfhydryl group in the protein.

Spectrophotometric titration of papain with *N*-pentylmaleimide indicated that only one functional group of the enzyme reacts with the maleimide (Table II). A 1:1 stoichiometry exists between the modification of this group and the loss of catalytic activity. On the basis of the stoichiometry of this reaction, the known reactivity of the papain sulfhydryl group, and the previously demonstrated selective alkylation of the papain sulfhydryl group (Balls and Lineweaver, 1939; Finkle and Smith, 1958; Hollaway *et al.*, 1964; Light, 1964; Sluyterman, 1968), it was concluded that under the conditions employed, the maleimide inactivation of papain results from the modification of the active-site sulfhydryl group. It is of interest in this respect that the substrate-competitive inhibitor, α-*N*-benzoyl-L-arginine does show protection against maleimide inactivation.

Studies of enzyme inactivation by *N*-alkylmaleimides have been used to investigate the nature of the environment of essential sulfhydryl groups of several enzymes. Studies of this nature have been reported for yeast alcohol dehydrogenase (Heitz *et al.*, 1968), hog kidney D-amino acid oxidase (Fonda and Anderson, 1969), and rabbit muscle L-α-glycerophosphate dehydrogenase (Anderson *et al.*, 1970). In each of these cases, *N*-alkylmaleimide inactivation of the enzyme was facilitated by increasing the chain length of the maleimide derivatives. Since there is no chain-length effect in the reactions of *N*-alkylmaleimides with cysteine and gluthathione (Heitz *et al.*, 1968), it has been suggested that the chain-length effects observed in enzyme inactivation by *N*-alkylmaleimides occur in the binding of these compounds to the enzyme prior to the inactivating sulfhydryl reaction. A second step, the reaction of bound maleimide with an essential sulfhydryl group, would lead to irreversible inactivation and would be insensitive to chain-length effects as in the case of other sulfhydryl–maleimide reactions. In studies of D-amino acid oxidase (Fonda and Anderson, 1969) saturation effects on rates of enzyme inactivation were observed and the same maximum rate of inactivation was approached at high concentrations of *N*-alkylmaleimides of different chain length.

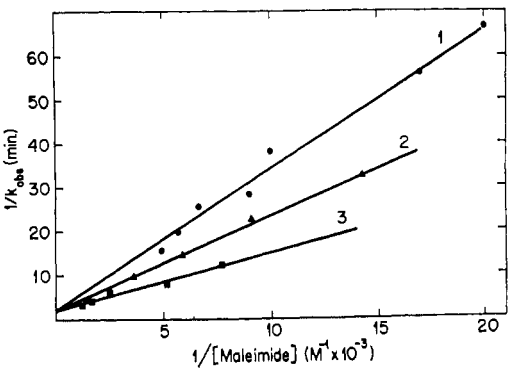

FIGURE 4: The relationship of the reciprocal of the pseudo-first-order rate constants of papain inactivation to the reciprocal of the *N*-alkylmaleimide concentration: line 1, *N*-butylmaleimide; line 2, *N*-pentylmaleimide, and line 3, *N*-hexylmaleimide.

In the present study, saturation effects on rates of inactivation of papain were observed (Figure 3) and evidence was presented (Figure 4) that the same maximum rate of inactivation was approached at high concentrations of *N*-butyl-, *N*-pentyl-, *N*-hexyl-, and *N*-heptylmaleimide. The disappearance of chain-length effects on the rate of inactivation at enzyme saturation indicates that these effects occur predominantly in the binding of maleimides to the enzyme. One can consider the following inactivation process where step

$$\text{enzyme} + \text{maleimide} \underset{k_2}{\overset{k_1}{\rightleftharpoons}}$$

$$\text{enzyme–maleimide complex} \xrightarrow{k_3} \text{inactive enzyme}$$

one represents a reversible binding of maleimide to the enzyme and step two (k_3) an irreversible inactivating reaction of the bound maleimide with the active-site sulfhydryl group. The rate constant (k_3) can be evaluated from the data of Figure 4 to be 0.5 min^{-1}. The pseudo-first-order rate constants (k_{obsd}) obtained at different concentrations of a given maleimide can be shown to be equal to $k_3 \times K_{assoc} \times$ [maleimide], where K_{assoc} is the equilibrium constant for the formation of enzyme–maleimide complex. Knowing k_3 and k_{obsd} at a given maleimide concentration, one can calculate K_{assoc}. The equilibrium (binding) constants for the different *N*-alkylmaleimides are shown in Figure 5 plotted on a semi-logarithmic scale against the number of carbons of the alkyl side chains of the maleimide derivatives. The change in free energy of binding ($\Delta\Delta F$) per methylene group can be calculated from the relationship

$$\Delta\Delta F = -2.303RT\,\Delta \log K_{assoc}$$

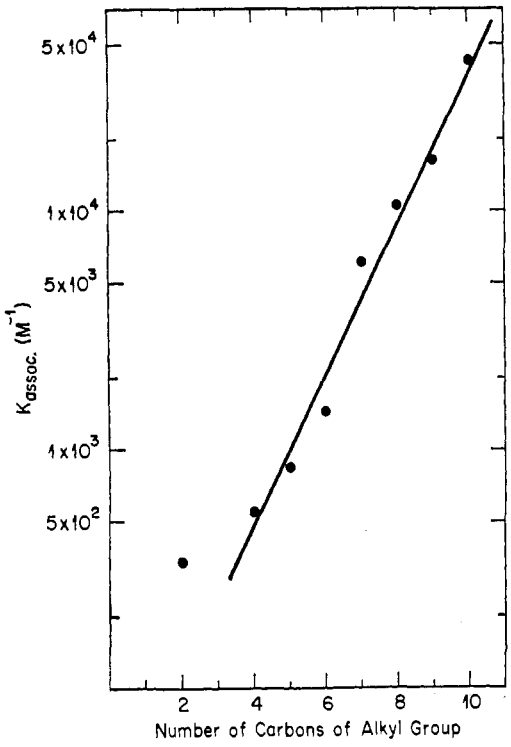

FIGURE 5: The relationship of the logarithm of the equilibrium constants for enzyme–maleimide complex formation to the number of carbons of the alkyl side chain of the maleimide derivatives.

In this manner, a free-energy change per methylene group of −0.434 kcal per mole was obtained. This value lies within the range suggested for interactions through dispersion forces (Webb, 1963).

X-Ray studies of crystalline papain (Drenth *et al.*, 1968) have demonstrated the active-site sulfhydryl group to be located in a nonpolar cleft region of the enzyme. The chain length facilitated inactivation of the enzyme by *N*-alkylmaleimides which can be minimized by the binding of a substrate-competitive inhibitor indicates that this nonpolar cleft region can influence reactions that occur with the active-site sulfhydryl group. In earlier studies of the nucleophilic attack of various amines on *trans*-cinnamoyl-papain (Brubacher and Bender, 1966), the higher rates of acyl transfer to tryptophanamide compared to those with glycinamide may have resulted from nonpolar interactions with the cleft region of the enzyme. Also, chain-length effects were observed (Glazer, 1966) in the inhibition of papain-catalyzed hydrolysis of glycylglycine esters by straight-chain alcohols.

The observation that the apparent second-order rate constant for papain inactivation by *N*-phenylmaleimide is essentially the same as that obtained for *N*-pentylmaleimide suggests that ring–ring interactions most likely do not play a role in facilitating inactivation. The inability of *N*-alkylmaleamic acids to inactivate the enzyme effectively is consistent with previous reports of poor binding of negatively charged inhibitors at neutral pH (Sluyterman, 1964; Chaiken and Smith, 1969b).

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