Stearn, A. E. (1949), *Adv. Enzymol. 9,* 25.
Wong, J. T. F., and Hanes, C. S. (1962), *Can. J. Biochem. Physiol. 40,* 763–804.
Yphantis, D. A. (1964), *Biochemistry 3,* 297–317.
Yue, R. H., Noltmann, E. A., and Kuby, S. A. (1967), *Biochemistry 6,* 1174–1183.
Yue, R. H., Noltmann, E. A., and Kuby, S. A. (1969), *J. Biol. Chem. 244,* 1353–1364.

Identity and Properties of the Chloride Effector Binding Site in Hog Pancreatic α-Amylase†

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ABSTRACT: The Cl^- activated α-amylase from mammalian sources has been shown previously to possess one Cl^- binding site per molecule (Levitzki, A., and Steer, M. L. (1974), *Eur. J. Biochem. 41,* 171). Upon binding of the Cl^- effector the k_{cat} of the amylolytic reaction is increased 30-fold whereas the affinity toward the substrate remains unchanged. In the study presented here we have identified the Cl^- binding site as a single ε-amino group of lysine. The pK of the unique amino group was found to be 9.1, significantly lower than the pH of a free ε-amino group of lysine. This ε-NH_2 group can be blocked by a 2,4-dinitrophenyl group upon treating the enzyme with 2,4-dinitrofluorobenzene at pH 7.9. The dinitrophenylamylase is devoid of Cl^- binding capacity but retains its substrate binding capacity. The dinitrophenylamylase also possesses the basal amylolytic activity characteristic of the unmodified Cl^- free enzyme, indicating that the catalytic machinery of the enzyme is not affected by dinitrophenylation. α-Limit dextrins and maltose which bind to the active site protect the enzyme against dinitrophenylation at least as effectively as the Cl^- effector. These observations indicate that the Cl^- binding lysyl residue is close to the active site and, upon binding, the Cl^- effector induces an enhancement in the catalytic efficiency.

Mammalian α-amylase is known to be activated 30-fold by Cl^- anions (Fischer and Stein, 1960, and references therein). It has been shown recently that Cl^- is an exclusive k_{cat} effector activating the enzyme 30-fold toward starch or the low molecular weight substrate *p*-nitrophenyl maltoside (Levitzki and Steer, 1974). The enzyme was shown to possess one chloride binding site per molecule of enzyme with dissociation constants of 3×10^{-4} M at 25 °C and 1.0×10^{-4} M at 4 °C. Chloride binding induces a subtle conformational change in the enzyme as reflected by the suppression of the exchange of 26 protons and a 240-fold increase in the amylase-Ca^{2+} binding constant, from 8.3×10^8 to 2×10^{11} M^{-1}. The conformational change is minor since it it not detected by spectroscopic measurements on the protein such as circular dichroism and fluorescence of tryptophan moieties or of specific probes attached to the enzyme. It is clear, therefore, that the large rate enhancement induced by the Cl^- effector is due to subtle conformational changes, probably confined to the region of the catalytic center.

In this study we have tried to probe the Cl^- binding site by physicochemical measurements and specific chemical modifications. Furthermore, attempts were made to gain more information concerning the interaction between the Cl^- binding site and the substrate binding site.

Materials and Methods

Undiluted acetone powder of hog pancreas was obtained from Marshall Division Laboratories, Miles. Maltose, sucrose, and lactose, all analytical grade, and shellfish glycogen were obtained from Merck. Soluble starch was obtained from British Drug House and diisopropyl fluorophosphate (iPr_2FP)[1] was from Sigma. $Na^{36}Cl$ and [^{14}C]N_2phF[1] were obtained from Radiochemical Centre. All other purchased chemicals used were of the highest analytical grade available. All solutions were prepared in Corning double distilled water. N^{ϵ}-Dinitrophenyllysine and $N^{\epsilon},N^{\epsilon'}$-dinitrophenylbislysine were prepared by Mr. Israel Jacobson from L-lysine·HCl (Ajimoto, Japan) by reaction with N_2pH-F in the presence of excess $CuCO_3$ according to published procedures (Sanger, 1946; Porter and Sanger, 1948; Greenstein and Winitz, 1961). *o*-Dinitrophenyltyrosine and imidazoledinitrophenylhistidine were kindly donated by Dr. Mati Fridlein from the Department of Organic Chemistry. *S*-Dinitrophenylcysteine was obtained from Mann.

α-Amylase. The enzyme was purified according to the method devised by Loyter and Schramm (1962) with slight modifications. The glycogen of the enzyme–glycogen complex was incubated for 2 h at 30 °C in 0.02 M Pipes[1] containing 3×10^{-3} M NaCl and 5×10^{-4} M $CaCl_2$, pH 6.9, to digest the glycogen. The α-limit dextrins were removed by treatment with Norit A charcoal at pH 8.0–8.5. The Norit A was previously treated with 1.0 N HCl, then with 0.01 M EDTA (pH 7.0) and then thoroughly washed with double distilled water. Enzyme concentration was determined spectrophotometrically using the value $E_{1\%}^{280} = 24.1$ (Hsiu et al., 1964) and the enzyme activity was measured according to Bernfeld (1955) but at 30 °C (Loyter

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[1] Abbreviations used are: N_2ph-F, 1-fluoro-2,4-dinitrobenzene; N_2ph-F_2, 1,5-difluoro-2,4-dinitrobenzene; N_2ph, dinitrophenyl; iPr_2-FP, diisopropyl fluorophosphate; pipes, piperazine-N,N′-bis(2-ethanesulfonic acid).

and Schramm, 1962). One unit of enzyme is defined as the amount of enzyme releasing 1 mg of maltose-hydrate equivalent from soluble starch during 3 min at 30 °C. The specific activity of the preparation used throughout the study was 1450 ± 50 units/mg. The enzyme was kept at 4 °C in 0.02 M Pipes containing 5×10^{-4} M $CaCl_2$, 3×10^{-3} M NaCl, 0.02% NaN_3, and 2 μg/ml of iPr_2FP. Under these conditions the enzyme remains fully active for a few months.

Cl^- Free Enzyme. Cl^- free enzyme is prepared by prolonged dialysis against 0.01 M sodium phosphate buffer (pH 6.9) and then against double distilled water. The enzyme obtained contains 0.03 Cl^- atom per enzyme molecule as determined amperometrically (Levitzki and Steer, 1974). Such a preparation exhibits 5-6% activity which is increased to 100% in the presence of Cl^- in the assay mixture. Removal of the rest of the Cl^- decreases the activity to the basal level of 3.5% as reported earlier (Levitzki and Steer, 1974).

Equilibrium Dialysis. Equilibrium dialysis to measure $^{36}Cl^-$ binding was performed as described earlier (Levitzki and Steer, 1974). The Cl^- binding curves were obtained at different pH values, using different buffers: Tris-maleate (pH 5.6), sodium phosphate (pH 6.0, 6.9, 7.4, 8.0), sodium borate (pH 8.2, 9.0) and glycyl-glycine-NaOH (pH 9.4, 9.8). The final concentration of the buffer was always 0.1 M.

Effect of EDTA. The Cl^- binding capacity of apoamylase was checked by conducting the equilibrium dialysis experiment using native α-amylase in the presence of 0.01 M EDTA. In the presence of 0.01 M EDTA at pH 7.0 and in the presence of iPr_2FP the enzyme retains its full activity as determined by the re-addition of Ca^{2+} in the enzyme assay (Steer and Levitzki, 1973).

Preparation of α-Limit Dextrins. α-Limit dextrins were prepared according to Levitzki (1963). Shellfish glycogen (500 mg) was dissolved in 100 ml of 0.01 M sodium phosphate buffer (pH 8.0). To the solution, 17.4 mg of pure α-amylase was added and the digestion was performed for 24 h at 4 °C in a dialysis bag immersed in the same buffer. The enzyme was precipitated by 5% Cl_3CCOOH. The Cl_3CCOOH supernatant was neutralized to pH 6.9 using NaOH and dialyzed extensively against double distilled water. The aqueous solution was lyophilized, dissolved in 2.5 ml of double distilled water, and chromatographed on a Sephadex G-25 column (1.2 × 42 cm) using double distilled water as the eluent. Four fractions were obtained and the average degree of polymerization was determined by the determination of total sugar (Dubois et al., 1956) and reducing sugar (Kidby and Davidson, 1973).

Dinitrophenylation of α-Amylase. Dinitrophenylation was conducted at 21 °C in the dark using Cl^- free amylase at a concentration of 0.5 to 1.0 mg/ml. The buffers used for the experiments were: 0.05 M sodium borate (pH 8.2) and 0.05 M sodium phosphate (pH 6.9, 7.4, 7.9, 8.0). The modification reaction was either conducted with nonradioactive N_2ph-F or [^{14}C]N_2ph-F (1.67×10^9 cpm/mmol) using N_2ph-F to enzyme molar ratios of 100, 400, and 1000. When the enzyme was treated with N_2ph-F_2 rather than N_2ph-F, the molar ratio used was 400 reagent to enzyme. Both reagents were dissolved in ethanol and their concentrations were determined by dissolving a sample in 1.0 N NaOH and measuring the optical absorbance. The extinction coefficients used were, for N_2ph-F, ϵ_m^{360} = 14 800 M^{-1} cm^- 1 and, for N_2ph-F_2, ϵ_m^{345} = 14 000 M^{-1} cm^{-1}. During dinitrophenylation enzyme activty was measured, at different times, both in the absence of Cl^- (basal activity) using Cl^- free substrate (Levitzki and Steer, 1974) and in the presence of Cl^- (maximal activity). The degree of enzyme modification was determined spectrophotometrically, measuring the absorbance of the modified enzyme at 360 nm for N_2ph incorporation or at 345 nm for N_2ph-F_2 reaction. The N_2ph incorporation values obtained spectrophotometrically were always checked against ^{14}C incorporation using [^{14}C]N_2ph-F_2. The concentration of modified α-amylase was checked by absorbance at 280 nm and by the micro-Lowry method (Lowry et al., 1951) using native α-amylase as a standard.

Stable Amylase-Glycogen Complex. The formation of a stable insoluble amylase-glycogen complex was obtained by mixing 2.0 mg of glycogen with different enzyme concentrations in Tris-HCl buffer (pH 8.5) in 40% (v/v) ethanol at 4 °C in a final volume of 2.8 ml. Under these conditions, in the absence of glycogen, α-amylase as well as N_2ph-modified amylase are fully soluble. Other proteins such as lysozyme or ovalbumin, used as controls, are also fully soluble in the 40% alcoholic solution.

Thin-Layer Chromatography (TLC) of N_2ph Amino Acids. Thin-layer chromatography on silica was performed to verify the purity of the synthesized N^ϵ-dinitrophenyllysine and N^ϵ,$N^{\epsilon'}$-dinitrophenylbislysine. Five solvent systems were used for this purpose: pyridine (0.2 M)-methanol (3:5); acetic acid-H_2O-pyridine (1:10:2); dioxane-H_2O-methanol-pyridine (0.2 M) (1:1:1:6:1); $CHCl_3$-methanol-acetic acid (19:1:0.6); 1-butanol-acetic acid-H_2O (4:1:5).

Characterization of Native Amylase and N_2ph-amylase. After total acid hydrolysis (6 N HCl, 110 °C 22 h in vacuo) of either native enzyme or modified enzyme the amino acid composition was determined using a Beckman automatic amino acid analyzer. The N_2ph amino acid markers used to identify the N_2ph amino acids obtained upon total acid hydrolysis were: *O*-dinitrophenyltyrosine, imidazoledinitrophenylhistidine, *S*-dinitrophenylcysteine, and N^ϵ-dinitrophenyllysine. TLC of N_2ph amino acids was performed on silica using four solvent systems (*sec*-butyl alcohol-acetic acid-H_2O (4:1:5); 2-propanol-NH_4OH (24% NH_3) (7:3); *tert*-amyl alcohol-acetic acid-chloroform (30:3:70); sodium phosphate buffer (0.03 M, pH 6.9).

Reduction and Carboxymethylation of N_2ph-amylase. The N_2ph enzyme was reduced with 10 equiv of dithiothreitol per enzyme SH in 0.2 M Tris-HCl (pH 8.0), in the presence of 8 M urea for 20 min in a stoppered test tube, in the dark at room temperature. Twenty minutes later 50 equiv of ICH_2COOH per enzyme SH was added and the mixture was incubated for 20 min. Then the enzyme was dialyzed against double distilled water. If precipitate was formed a drop of 5 N NaOH was added. The material was then lyophilized. Reduction and carboxymethylation yielded the same results when conducted in 0.1 M *N*-ethylmorpholine (pH 8.6) in the presence of 0.01 M EDTA. N_2ph incorporation into the modified enzyme was measured spectrophotometrically at 360 nm and checked against the incorporation of [^{14}C]N_2ph.

Results

Effect of pH on $^{36}Cl^-$ Binding. The effect of pH on $^{36}Cl^-$ binding to Cl^- free α-amylase was determined between pH 5.6 and 9.5 using equilibrium dialysis at 4 °C (Figure 1). Independently we can show that the enzyme remains fully active even for periods longer than that required

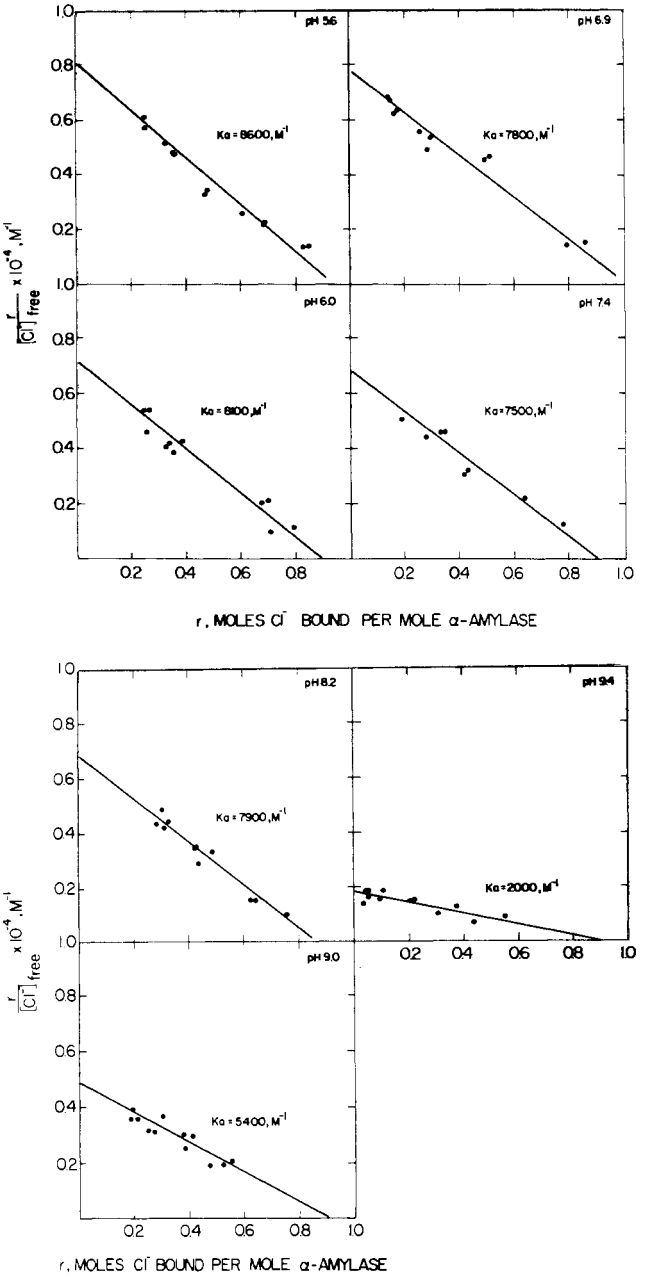

FIGURE 1: The binding of $^{36}Cl^-$ by α-amylase as a function of pH. The binding of $^{36}Cl^-$ was measured by equilibrium dialysis at 4 °C as described in the Materials and Methods section.

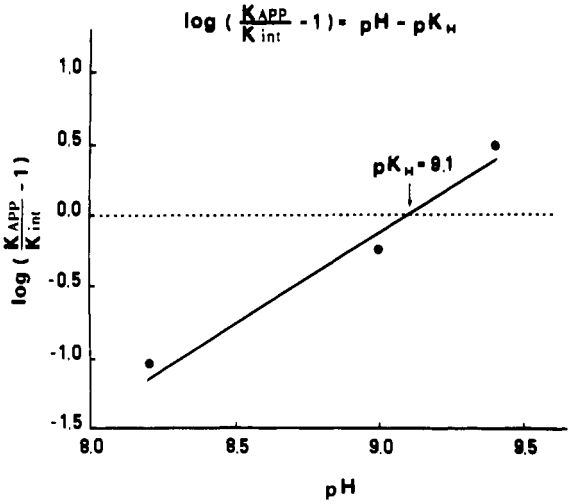

FIGURE 2: The pK_H of the Cl^- binding group. The data of Figure 1 were plotted according to eq 1 in the text: $\log (K_{app}/K_{int}) - 1 = pH - pK_H$, where K_{app} is the measured amylase-Cl^- dissociation constant at each pH, and K_{int} is the intrinsic amylase Cl^- dissociation constant measured.

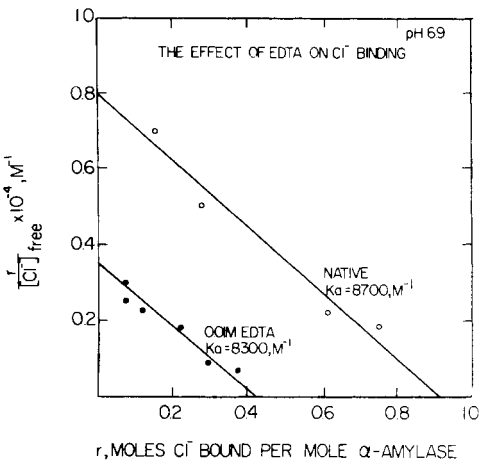

FIGURE 3: The effect of EDTA on $^{36}Cl^-$ binding. The binding of $^{36}Cl^-$ was measured at pH 7.0 in sodium phosphate buffer at 4 °C in the presence of 0.01 M EDTA.

for an equilibrium dialysis experiment. At pH values higher than 9.5, the enzyme loses activity irreversibly; thus, no measurements of Cl^- binding were performed at pH values higher than 9.4. The affinity of the enzyme toward Cl^- decreases as a function of pH (Figure 1) without a change in the total number of Cl^- binding sites, which is consistently found to be one per enzyme molecule. The dependence of the apparent Cl^- amylase association constant on pH can be analyzed according to eq 1 and plotted in Figure 2,

$$\log [(K_{app}/K_{int}) - 1] = pH - pK_H \quad (1)$$

where K_{app} is the measured association constant and K_{int} is the intrinsic amylase-Cl^- association constant measured at low pH, much below the pK_H of the ionized group which binds the anion. The basic assumption underlying eq 1 is that only the ionized form of the group binds the Cl^- anion. From Figure 2 it is seen that pK_H is 9.1.

The Inability of Ca^{2+}-Free Apoamylase to Bind Cl^-. In the presence of EDTA amylase is incapable of binding Cl^- (Figure 3). In the presence of EDTA equilibrium 2 is estab-

$$EnzCa(II) + EDTA \rightleftharpoons Enz + CaEDTA \quad (2)$$

lished (Levitzki and Steer, 1974). The apoenzyme is incapable of binding Cl^- but the remaining native enzyme (EnzCa(II)) in the equilibrium mixture continues to bind Cl^- with the same affinity constant but the overall Cl^- to amylase stoichiometry decreases as a function of increasing EDTA (Figure 3). It can be calculated (see Appendix) that the fraction on native enzyme in the enzyme-EDTA mixture matches the fraction of $^{36}Cl^-$ occupancy measured directly (Figure 3). This establishes that Ca^{2+}-free α-amylase does not bind Cl^- in a measurable affinity. Thus, for example, at pH 6.9, in the presence of 0.01 M EDTA, it can be calculated from the equilibrium constant for eq 2 (Levitzki and Steer, 1974) that the fraction of apoenzyme is 0.65. The experimental value for an enzyme species incapable of binding $^{36}Cl^-$ under these conditions was found to be 0.56 (Figure 3).

The Absence of F^- and Acetate Binding. α-Amylase was

Table I: The Modification of α-Amylase by N_2ph-F and Its Products.

Degree of Modification (mol of N_2ph Bound per mol of Enzyme)	Unmodified Amino Acids[c]			Modified Amino Acids[d]		
	Lys	His	Arg	Lys	His	Arg
2.10 to 3.8[a,b]	16.80	8.86	27.0	3.2	0.0	0.0
3.0 and higher[f]					no modification	
Native α-amylase	19.58	9.03	26.90			
Theor. values[e]	20.0	9.0	27.0			

[a] By spectral measurements, enzyme 5% active in the presence of Cl^-. [b] By [^{14}C]N_2ph incorporation. [c] The preparation yielding the value was examined by amino acid analysis. [d] By amino acid analysis. [e] From Caldwell et al. (1954). [f] Only lysine residues are modified.

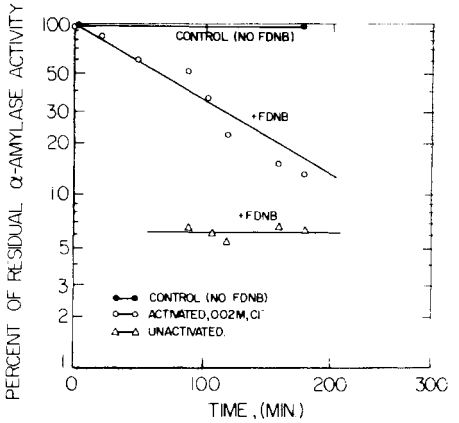

FIGURE 4: The independence of basal activity from the extent of dinitrophenylation. The dinitrophenylation, using N_2ph-F, was conducted as described under Materials and Methods, in the absence of Cl^-. The extent of dinitrophenylation was measured either spectrophotometrically or by using [^{14}C]N_2ph-F as described under Materials and Methods. The two techniques agreed extremely well. Cl^- free activity was measured using thoroughly dialyzed soluble starch.

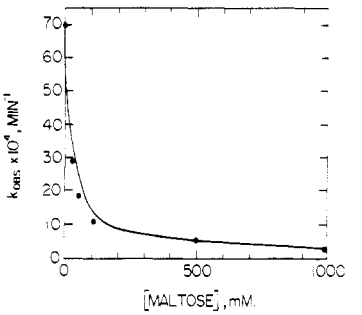

FIGURE 5: The dependence of the inactivation constant k_{obsd} on maltose concentration. The values for k_{obsd} were calculated using the relation $t_{1/2} = \ln 2/k_{obsd}$, where $t_{1/2}$ is the inactivation of half-life.

found not to bind F^- or acetate at any measurable affinity. Furthermore, the affinity of α-amylase toward $^{36}Cl^-$ was found to be unaltered in the presence of either 0.05 M NaF or CH_3COONa, indicating that the affinity toward these ions is negligible.

The Inhibition of α-Amylase by N_2ph-F. N_2ph-F was found to inactivate α-amylase activity and the standard conditions chosen for dinitrophenylation of the enzyme were pH 7.9 and solution conditions as described in the Materials and Methods section. The molar ratio of N_2ph-F to enzyme used was 400 to 1.0. The rate of dinitrophenylation was found to be first order; thus the rate constant, k_{obsd}, for enzyme inactivation was computed from the half-life value for the inactivation reactions. The inactivation pattern was found to be independent of whether the reaction was conducted in a phosphate or borate buffer. It was found that, whereas Cl^- stimulated activity was inhibited, the "basal" Cl^- free activity was independent of dinitrophenylation (Figure 4).

Degree of Dinitrophenylation. The degree of enzyme modification by N_2ph-F, in the absence of Cl^-, was examined by three independent procedures. The dinitrophenylated enzyme was reduced and carboxymethylated and the number of bound N_2ph groups was determined either by the incorporation of [^{14}C]N_2ph or spectrophotometrically. Furthermore, the number of N_2ph groups attached was found to be identical in an uncarboxymethylated vs. reduced carboxymethylated enzyme. This finding demonstrates that no cysteine residues were modified (Shaltiel, 1967). The two SH groups in α-amylase can be modified without the loss of Cl^- dependent activity (Steer et al., 1974). These groups remain intact after N_2ph-F treatment. The degree of dinitrophenylation was also examined by amino acid analysis of the hydrolyzed protein and three lysine residues were found to be modified (Table I).

The Modification of α-Amylase by N_2ph-F_2. The Cl^- stimulated activity of α-amylase is inhibited 95% upon the modification of six lysine residues by N_2ph-F_2. No $N^{\epsilon},N^{\epsilon'}$-dinitrophenylbislysine was detected upon total acid hydrolysis. The rate of enzyme inactivation by N_2ph-F_2 is identical with that by N_2ph-F.

The Inhibition of Dinitrophenylation by α-Limit Dextrins. α-Limit dextrins (degree of polymerizations = 25) inhibit the rate of enzyme modification with N_2ph-F. At 5×10^{-5} M enzyme and 4.7×10^{-5} M dextrins the pseudo-first-order inactivation constant is 5.72×10^{-3} min^{-1} as compared to 6.9×10^{-3} min^{-1} in the absence of dextrins. Under the experimental conditions used soluble enzyme-dextrin complexes are formed (Loyter and Schramm, 1966).

The Inhibition of Enzyme Dinitrophenylation by Maltose. Maltose inhibits the rate of α-amylase inactivation by N_2ph-F. The dependence of the pseudo-first-order inactivation rate constant, k_{obsd}, on maltose concentration is depicted in Figure 5. It can be shown that the first-order inactivation constant k_{obsd} is inversely proportional to the maltose concentration (Figure 6) according to eq 3, where k_1 and k_2 are the pseudo-first-order constants for enzyme and enzyme-maltose inactivation. K_D is the enzyme-maltose dissociation constant. The enzyme possesses two lysine residues which can be labeled with [^{14}C]N_2ph-F in the absence of maltose concomitant with the loss of 90% of the enzyme activity. In the presence of 1.0 M maltose only one lysine residue becomes labeled with almost no loss of activity. The inhibition constant for maltose in the amylase reaction was

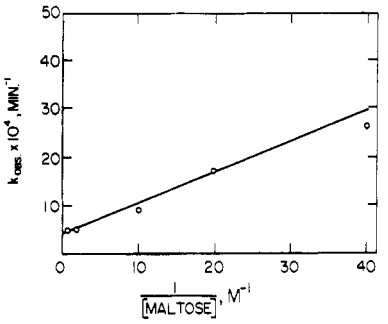

FIGURE 6: The dependence of the inactivation constant on the maltose reciprocal concentration. Data of Figure 5 are plotted according to eq 3 of the text: $k_{obsd} = k_1 + (k_2K_D/[\text{maltose}])$, where k_1 is the pseudo-first-order rate constant of enzyme inactivation and k_2 is the pseudo-first-order rate constant of the enzyme-maltose complex. K_D is the enzyme-maltose dissociation constant.

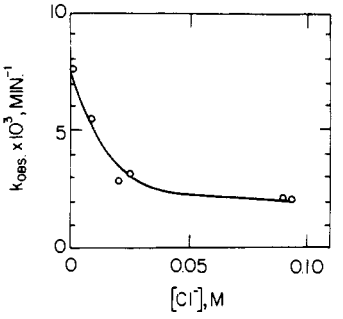

FIGURE 7: The dependence of the inactivation constant k_{obsd} on Cl^- concentration. The values for k_{obsd} were calculated using the relation $t_{1/2} = \ln 2/k_{obsd}$.

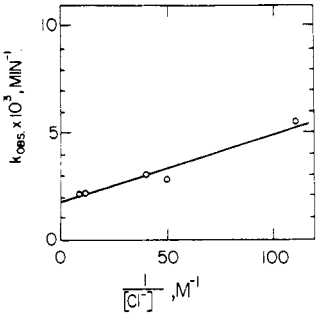

FIGURE 8: The dependence of the inactivation on the Cl^- reciprocal concentration. Data of Figure 7 were plotted according to the equation: $k_{obsd} = k_1 + (k_2K_D/[Cl^-])$ where k_1 is the pseudo-first-order rate constant for enzyme inactivation and k_2 is the pseudo-first-order rate constant for enzyme-Cl^- inactivation. K_D is the measured enzyme-Cl^- dissociation constant.

found to be 4.6×10^{-3} M in constrast to the value 2.5×10^{-2} M reported by Elodi (1972). The value reported here is based on the measurement of the appearance of reducing groups and that reported by Elodi is based on an iodine assay which measures the disappearance of high molecular weight substrate. Thus, the number reported here measures more accurately the true dissociation constant. The effect of maltose is specific since sucrose and lactose have no effect.

$$k_{obsd} = k_1 + k_2K_D/[\text{maltose}] \quad (3)$$

The Inhibition of Enzyme Dinitrophenylation by Cl^-. The rate of enzyme modification by N_2ph-F is inhibited by Cl^- (Figure 7). As in the case of maltose, the first-order inactivation constant, k_{obsd}, is inversely proportional to the

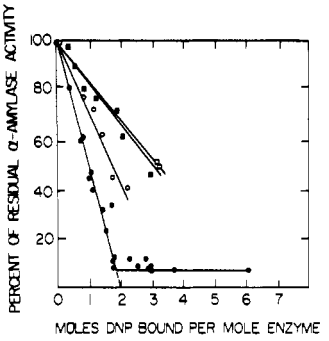

FIGURE 9: The extent of enzyme inactivation as a function of the extent of modification. The enzyme was incubated with N_2ph-F under the standard conditions for dinitrophenylation (see Materials and Methods). Different Cl^- concentrations were incorporated in the dinitrophenylation mixture: no Cl (●), 0.009 M Cl^- (○), 0.045 M Cl (□), and 0.094 M Cl (■). The degree of enzyme modification was measured spectrophotometrically at 360 nm or by the use of [^{14}C]N_2ph-F (see Materials and Methods). All the N_2ph groups were shown to appear as N^{ϵ}-dinitrophenyllysine (see text).

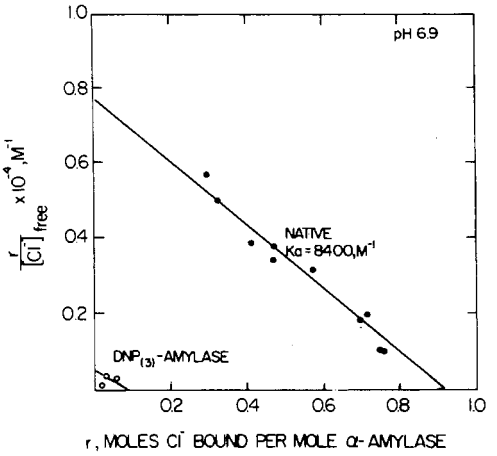

FIGURE 10: The absence of $^{36}Cl^-$ binding by $(N_2ph)_3$-amylase. The extent of $^{36}Cl^-$ binding was measured by equilibrium dialysis as described in the text.

concentration of Cl^- (Figure 8). The first-order kinetic constants characterizing the rate of enzyme inactivation by N_2ph-F are 1.6×10^{-3} min^{-1} for the free enzyme (k_1) and 1.03×10^{-1} min^{-1} for the enzyme-Cl complex (k_2) and 0.6×10^{-1} min^{-1} for the enzyme-maltose complex (k_2). The inactivation of the enzyme by N_2ph-F is due exclusively to the dinitrophenylation of lysine residues (Table I). In the presence of Cl^- more lysine residues become modified before the enzyme is inactivated, probably due to the slowing down of the dinitrophenylation reaction of the essential lysine residue (see Discussion), thus allowing more lysine substitution during the slow course of enzyme-Cl^- inactivation (Figure 9).

The Inability of $(N_2ph)_3$-amylase to Bind $^{36}Cl^-$. The dinitrophenylated derivative of α-amylase which possesses "basal" activity (Figure 9) is incapable of binding the effector $^{36}Cl^-$ (Figure 10).

The Ability of $(N_2ph)_3$-amylase to Bind Glycogen. Glycogen is known to precipitate α-amylase selectively in the cold in the presence of 40% ethanol (Loyter and Schramm, 1962). Glycogen was found to precipitate $(N_2ph)_3$-amylase as well as native amylase. For comparison we could demonstrate that proteins such as ribonuclease lyzozyme and ovalbumin are not precipitated under identical conditions.

Table II: TLC of Total Hydrolysate of $(N_2ph)_n$-amylase.

N_2ph-Amino Acid	Solvent System			
	Butanol-HOAc-H_2O (4:1:5)	2-Propanol-NH_4OH (24% NH_3) (7:3)	*tert*-Amyl alcohol-HOAc-$CHCl_3$(30:3:70)	Sodium Phosphate Buffer (0.02 M, pH 6.9)
ϵ-N_2ph-Lys	0.47	0.52	0.055	0.38
Im-N_2ph-His	0.38	0.70	No migration	0.53
O-N_2ph-Tyr	0.72	0.78	0.92	
S-N_2ph-Cys	0.49	0.60	No migration	
$(N_2ph)_1$-α-amylase hydrolysate	0.48	0.51	0.055	0.38
$(N_2ph)_3$-α-amylase hydrolysate	0.48	0.52	0.055	0.38

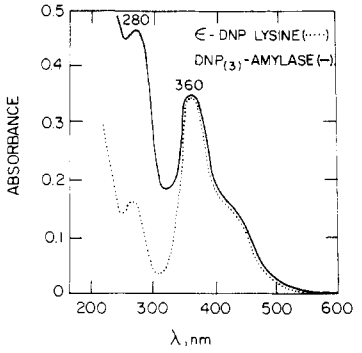

FIGURE 11: The spectral properties of $(N_2ph)_3$-amylase as compared to N^{ϵ}-dinitrophenyllysine. The spectra of $(N_2ph)_3$-amylase and of N^{ϵ}-dinitrophenyllysine at equal N_2ph concentration are shown.

The Characterization of N_2ph-amylase. Upon total acid hydrolysis and subsequent TLC of the products, only dinitrophenyllysine was shown to be formed. TLC was conducted using a variety of solvent systems (see Materials and Methods). Representative results are depicted in Table II. Even higher degrees of dinitrophenylation (Figure 9) result in dinitrophenyllysine upon total acid hydrolysis. In each case it can be shown that the number of lysine residues missing in the acid hydrolysate can be accounted for as dinitrophenyllysine. No modifications of tyrosine or histidine occur (Table I). Upon reduction of the N_2ph enzyme by a 350-fold molar excess of β-mercaptoethanol at pH 8.0 no release of N_2ph groups occurs (Shaltiel, 1967) indicating that neither of the two SH groups becomes modified. Finally, the spectrum of $(N_2ph)_3$-amylase is identical with that of ϵ-dinitrophenyllysine (Figure 11).

The Effect of Cl^- on the Products of Dinitrophenylation. Cl^- inhibits the rate of enzyme modification (see above) but does not alter the type of amino acid (lysine) modified. In the presence of Cl^- higher degrees of dinitrophenylation occur before total inhibition occurs. The only amino acid modified under the conditions of dinitrophenylation was found to be lysine (Tables I and II).

Discussion

The Identity of the Cl^- Binding Site. The dependence of Cl^- binding on pH suggests a group with a pK_H = 9.1 as the binding site for Cl^- (Figures 1 and 2). The absence of an α-amino terminal in this protein (Fischer and Stein, 1960), as was also verified in the sole formation of ϵ-dinitrophenyllysine upon dinitrophenylation (Table I), suggested immediately a lysine residue. A pK_H of 9.1 is quite low for a lysine residue but a positively charged microenvironment can induce a decrease of the pK_H of an ϵ-NH_2 from 10.28 (Greenstein and Winitz, 1961) to 9.1. Such a positively charged environment can be provided, for example, by a Ca^{2+} atom which is bound tightly to the enzyme. The fact that a lysyl residue constitutes the Cl^- binding site is firmly established in the dinitrophenylation experiments described in detail. The dinitrophenylation abolishes Cl^- binding (Figure 10) as well as Cl^- dependent activity (Figures 4 and 9) but does not abolish Cl^- free activity (basal activity, Figures 4 and 9) or substrate binding. In the presence of a high concentration of the competitive inhibitor, maltose, one lysine residue is dinitrophenylated by N_2ph-F without a significant loss of activity. Thus, this lysine residue is not involved in Cl^- binding. In the absence of maltose or chloride full loss of activity occurs concomitantly with the labeling of *two* lysine residues (Figure 9). Also, the bifunctional reagent N_2ph-F_2 inhibits α-amylase activity with the same kinetics as N_2ph-F, indicating that the Cl^- binding site is not composed of two lysine residues. Thus, two lysine residues seem to have similar reactivity toward N_2ph-F but only one of them constitutes the Cl^- binding residue.

Properties of N_2ph-amylase. The dinitrophenylation with N_2ph-F yields $(N_2ph)_n$-amylase; for any n value we have shown that the only amino acid derivatized is lysine. The $(N_2ph)_{2-6}$-amylase is devoid of a $^{36}Cl^-$ binding site as measured by direct binding studies (Figure 10) but retains the full substrate binding capacity. The activity of the derivatized enzyme $(N_2ph)_n$-amylase is 3–5% for n = 2–6. The basal activity is independent of Cl^- (Figure 9) and is in fact very close to the basal activity of the native enzyme which is 3.5% (Levitzki and Steer, 1974). One can, therefore, conclude that blocking a single lysine inhibits the Cl^- binding and the Cl^- dependent activity, but does not abolish the basal Cl^- independent activity, or the capacity to bind substrate. These findings demonstrate that the catalytic machinery of the enzyme remains intact subsequent to dinitrophenylation, but cannot be modulated by Cl^- since its binding residue is blocked.

The Relationship between the Cl^- Site and the Active Site. Maltose and Cl^- were found to inhibit markedly the rate of enzyme dinitrophenylation by almost two orders of magnitude (Figures 5–8) and the species enzyme-Cl^- and enzyme-maltose have almost the same reactivity toward N_2ph-F. This finding may be taken as an indication that the Cl^- binding lysine residue is within the substrate binding site. An alternative possibility would be that a crucial lysine residue plays a key role in the establishment of the Cl^- binding site without participating directly in effector bind-

ing. However, the fact that both Cl^- and the inhibitors maltose and α-limit dextrins inhibit to the same extent N_2ph-F modification tends to favor the conclusion that the amino group of lysine is an integral part of the Cl^- binding site. The inhibition of the N_2ph-F reaction by α-limit dextrins is even more efficient than maltose: at 4.7×10^{-5} M dextrin and 5×10^{-5} M enzyme the N_2ph-F reaction is inhibited more strongly than by maltose at much higher concentrations. α-Limit dextrins bind tightly to α-amylase (Levitzki and Schramm, 1963; Levitzki et al., 1964) as compared to maltose; thus, it is not surprising that they exert their inhibitory effect at lower concentrations. Since the substrate or α-limit dextrins do not alter the affinity of α-amylase toward Cl^- (Levitzki and Steer, 1974), the fact that the substrate-like compounds render the lysine less reactive toward N_2ph-F may mean that the lysyl residue moves into a cleft, probably the active site, becomes inaccessible to N_2ph-F, but retains its accessibility to the anion effector. This movement of the lysyl residue is not accompanied by a change in the Cl^- binding affinity but seems to be essential for the 30-fold improvement in k_{cat}.

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Appendix: The Effect of Ca^{2+} on $^{36}Cl^-$ Binding

In a system in which native amylase is mixed with EDTA and the binding of $^{36}Cl^-$ is measured one would like to calculate how much of the ligand is bound to the native enzyme and how much is bound to the Ca^{2+}-free protein. Let us assume that Ca^{2+} increases 240-fold the affinity of the apoenzyme toward Cl^-, as it was found that Cl^- increases the affinity of amylase toward Ca^{2+} (Levitzki and Steer, 1974). Thus, the ratio between the two affinity constants K_1^1 and K_2^1 for the Ca^{2+}-enzyme and the apoenzyme, respectively, will be given by:

$$\frac{K_1^1}{K_2^1} = \frac{\dfrac{r_1}{N_1 - r_1}\dfrac{1}{[Cl]_{free}}}{\dfrac{r_2}{(N_2 - r_2)}\dfrac{1}{[Cl]_{free}}} = \frac{r_1(1 - r_2)}{r_2(1 - r)} = 240$$

$$N_1 = N_2 = 1$$

r_1 and r_2 are the fractions of the chloride-bound Ca^{2+}-enzyme and chloride-bound apoenzyme, respectively. N_1 and N_2 are the maximal number of Cl^- binding sites on each species.

It is clear from the equation that for any measured value of r_1, r_2 will be negligible (less than 1%) as compared to r_1 and r_{obsd} will always be almost identical with r_1. Thus, a $^{36}Cl^-$ binding experiment in the presence of EDTA actually measures the concentration of Ca^{2+}-enzyme in the system. At a certain concentration of EDTA or EGTA in the system, the amount of Ca^{2+}-free enzyme can be calculated from the known Ca-EDTA and Ca-enzyme association constants (Levitzki and Steer, 1974). This amount can then be compared to the number of $^{36}Cl^-$-binding sites measured in the presence of EDTA. It is found that, indeed, the number of $^{36}Cl^-$ binding sites matches the amount of Ca^{2+}-enzyme in the Ca^{2+}-EDTA mixtures.

References

Bernfeld, P. (1955), *Methods Enzymol. 1,* 149.
Caldwell, M. L., Dickey, F. S., Hanrahan, V. M., Kung, H. C., Kung, J. T., and Misho, M. (1954), *J. Am. Chem. Soc. 76,* 143.
Dubois, K. A., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28,* 350.
Elodi, P. (1972), *Acta Biochim. Biophys. Acad. Sci. Hung. 7,* 241.
Fischer, E. H., and Stein, E. A. (1960), *Enzymes, 2nd Ed., 4,* 313.
Greenstein, J. P., and Winitz, M. (1961), in Chemistry of Amino Acids, Vol. 1, New York, N.Y., Wiley, p 486.
Hsiu, J., Fischer, E. H., and Stein, E. A. (1964), *Biochemistry 3,* 61.
Kidby, D. K., and Davidson, D. J. (1973), *Anal. Biochem. 55,* 321.
Levitzki, A. (1963), M.Sc. Thesis, The Hebrew University of Jerusalem.
Levitzki, A., and Schramm, M. (1963), *Bull. Res. Counc. Isr., Sect. A 11,* 258.
Levitzki, A., Heller, J., and Schramm, M. (1964), *Biochim. Biophys. Acta 81,* 101.
Levitzki, A., and Steer, M. L. (1974), *Eur. J. Biochem. 41,* 171.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265-280.
Loyter, A., and Schramm, M. (1962), *Biochim. Biophys. Acta 65,* 200.
Loyter, A., and Schramm, M. (1966), *J. Biol. Chem. 241,* 2611.
Porter, R. R., and Sanger, F. (1948), *Biochem. J. 42,* 287.
Sanger, F. (1946), *Biochem. J. 39,* 507.
Shaltiel, S. (1967), *Biochem. Biophys. Res. Commun. 29,* 179.
Steer, M. L., and Levitzki, A. (1973), *FEBS Lett. 31,* 89.
Steer, M. L., Tal, N., and Levitzki, A. (1974), *Biochim. Biophys. Acta 334,* 389.