Reaction of an Aldolase–Substrate Intermediate with Tetranitromethane*

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ABSTRACT: The suitability of applying selective reagents to the detection and identification of enzyme–substrate intermediates has been examined using tetranitromethane (TNM) and aldolase of rabbit muscle. The rate of production of nitroform (ϵ_{350} 14,400) from TNM on addition to aldolase is markedly enhanced by the presence of substrates. Kinetic studies have demonstrated that this phenomenon is due to a reaction of TNM with an aldolase–substrate complex. The rate of nitroform production is directly proportional to the concentration of active enzyme.

The relationship between the rate of increase in A_{350} and substrate concentration reflects apparent Michaelis–Menten kinetics. The substrate concentration resulting in half-maximal rate of nitroform production (K_m') is virtually identical with the values of K_m for fructose 1,6-diphosphate and fructose 1-phosphate determined enzymatically. Phosphate competitively inhibits the reaction ($K_i' = K_i$). The consumption of substrate as a function of time indicates that TNM reacts with the substrate moiety of the enzyme–substrate complex. Comparison of the kinetics of nitroform production by native and carboxypeptidase-treated aldolase with different substrates indicates that the dihydroxyacetone phosphate–aldolase carbanion is the most likely TNM-reactive species. The carbanion specificity of TNM has been demonstrated also with a series of model systems. The potential usefulness of TNM in elucidating enzymatic mechanisms involving carbanion intermediates is discussed.

While chemical modification with specific reagents has been widely employed to identify functional residues of enzymes, its application to the detection and identification of enzyme–substrate intermediates has not been emphasized. This approach assumes that enzyme–substrate complexes would exhibit unique reactivities. In addition it would require reagents specific toward particular activated intermediates and its usefulness would be appreciably enhanced if the interaction could be detected readily and quantitatively. The characteristics of the reaction between tetranitromethane (TNM)[1] and aldolase in the presence of substrates seem to fulfill these criteria.

TNM has proven convenient as a modifying reagent for proteins. It nitrates tyrosyl residues (Riordan *et al.*, 1966, 1967; Sokolovsky *et al.*, 1966) and oxidizes sulfhydryl groups (Riordan and Christen, 1968). However, its properties suggest that it would also be a suitable reagent for modifying enzyme–substrate intermediates especially those with nucleophilic character, *e.g.*, carbanions. Its reaction can readily be detected by virtue of a yellow by-product, nitroform, with ϵ_{350} 14,400 (Glover and Landsman, 1964).

Carbanionic intermediates are known to occur in the enzymatic reactions of aldolase (Rose *et al.*, 1965). Previously we studied the reaction of TNM with aldolase in the absence of substrate. The reagent inactivated this enzyme due to oxidation of sulfhydryl groups. Addition of substrate, while protecting against inactivation, markedly increased the rate of nitroform production, suggesting a reaction between TNM and an enzyme–substrate complex (Riordan and Christen, 1968). The data presented here verify that TNM, indeed, reacts with an aldolase–substrate complex. Comparative studies with carboxypeptidase-treated aldolase and with nonenzymatic model systems indicate that the particular TNM-reactive intermediate is the carbanion of the dihydroxyacetone phosphate moiety of the enzyme–substrate complex.

Materials

Fructose 1,6-diphosphate aldolase from rabbit muscle, α-glycerophosphate dehydrogenase, triosephosphate isomerase, carboxypeptidase A, fructose 1,6-diphosphate tetracyclohexylammonium salt, fructose 1-phosphate dicyclohexylammonium salt, NADH, tetranitromethane, 2-mercaptoethanol, and Bio-Gel P-4 were obtained as reported previously (Riordan and Christen, 1968); α-chymotrypsin was obtained from Worthington Biochemical Corp.; bovine serum albumin from Armour and Co.; pyridoxal hydrochloride, pyridoxamine, fructose, fructose 6-phosphate, dihydroxy-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: TNM, tetranitromethane; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; GA, glyceraldehyde.

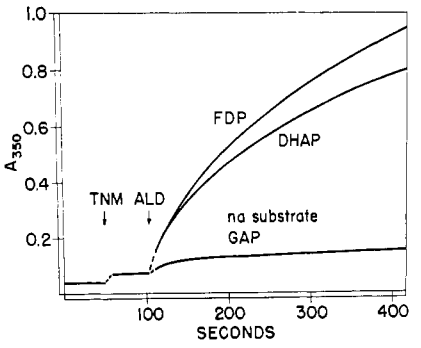

FIGURE 1: Production of nitroform by degradation of TNM in the presence of aldolase and its substrates. TNM (0.84 μmole) (25 μl of a 1:250 diluted solution of TNM in 95% ethanol) was added to 1.95 ml of 0.05 M Tris-Cl–0.3 M NaCl (pH 8.0), 25°, containing 1 mM FDP, 2 mM DHAP, 1.8 mM GAP, or no substrate, respectively. At the indicated time, 6.95×10^{-4} μmole of aldolase (20 μl of a 5.5-mg/ml solution) was added. The reactions were carried out in 1-cm cuvets and were followed by recording the increase in absorbance at 350 mμ.

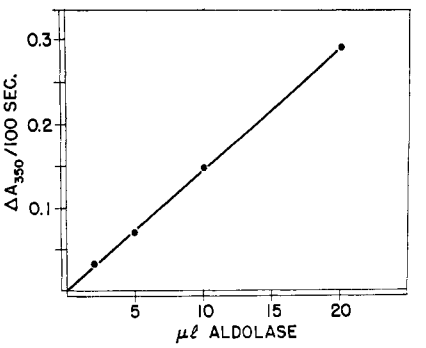

FIGURE 2: Rate of nitroform production at constant substrate concentration as a function of aldolase concentration. Aliquots of a solution of aldolase (5.5 mg/ml) were added to 1.95 ml of 1.2×10^{-5} M DHAP, containing TNM as in Figure 1.

acetone, and DL-glyceraldehyde from Nutritional Biochemical Corp.; thiamine hydrochloride from Calbiochem; glucose 1-phosphate dipotassium salt from General Biochemicals; glucose 6-phosphate sodium salt from Sigma Chemical Co.; isopropenyl acetate and glutamic acid from Eastman Organic Chemicals; and acetone from Merck.

Dihydroxyacetone phosphate dimethyl ketal dicyclohexylammonium salt (Boehringer) was converted into dihydroxyacetone phosphate by the method of Ballou and Fischer (1956).

DL-Glyceraldehyde 3-phosphate diethyl acetal barium salt (Boehringer) was treated with Dowex 50 (hydrogen form) and hydrolyzed at pH 2.1, 40°, for 48 hr, then neutralized to pH 7.4 with dilute NaOH. The concentrations of the dihydroxyacetone phosphate and glyceraldehyde 3-phosphate solutions were determined enzymatically with α-glycerophosphate dehydrogenase and triosephosphate isomerase.

Methods

Aldolase was dialyzed before use against the appropriate buffers. Its concentration and enzymatic activity were determined as reported previously (Riordan and Christen, 1968). The molecular weight of aldolase is 158,000 (Kawahara and Tanford, 1966).

The rate of production of nitroform was measured spectrophotometrically following the increase of absorbance at 350 mμ. A Unicam SP-800 recording spectrophotometer with a cell holder maintained at 25° was used. The reaction was initiated by the addition of enzyme to the otherwise complete reaction mixture in the spectrophotometer cuvet. After mixing, absorbance was recorded immediately. The rates of nitroform production reported are initial rates and were determined by obtaining the slope of the tangent to the absorbance *vs.* time curve 6–8 sec after addition of the enzyme (ϵ_{350} nitroformate, 14,400 M^{-1} cm^{-1}; Glover and Landsman, 1964). All rates are corrected for the nitroform production induced by the enzyme alone in the absense of substrate (Figure 1).

Results

On addition of aldolase to a solution of TNM in buffer or in buffer containing glyceraldehyde phosphate (GAP), there is a small increase in absorbance at 350 mμ due to oxidation and nitration of the protein and to nonspecific breakdown of TNM catalyzed by the basic groups of the protein (Riordan and Christen, 1968). However, when aldolase is added to a mixture containing either fructose 1,6-diphosphate (FDP) or dihydroxyacetone phosphate (DHAP) together with TNM, a marked increase in the rate of nitroform production is observed (Figure 1). Minimal spontaneous decomposition of TNM occurs in 0.05 M Tris-Cl–0.3 M NaCl buffer at pH 8.0 as judged by the lack of absorbance at 350 mμ. Similarly, there is no change in absorbance when TNM is added to solutions of FDP, DHAP, or GAP in the same buffer.

The enhanced production of nitroform takes place only with FDP, fructose 1-phosphate (F-1-P), or DHAP, but not with GAP, glyceraldehyde (GA), glucose 1-phosphate, glucose 6-phosphate, fructose, fructose 6-

TABLE I: Inactivation of Aldolase by TNM. Concomitant Decrease in Rate of FDP Cleavage and Nitroform Production in the Presence of Substrate.[a]

Per Cent of Initial Rate	
FDP Cleavage	Nitroform Production in the Presence of FDP
73	75
16	23
2	5

[a] Aldolase, partially inactivated by TNM, was prepared as described previously (Riordan and Christen, 1968). Conditions for the reaction with TNM are as in Figure 1.

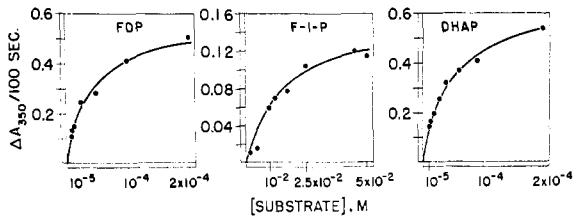

FIGURE 3: Rate of nitroform production by aldolase at varying substrate concentrations. The reaction mixture contained substrate at the indicated concentrations; the concentration of TNM and of aldolase and all other conditions were the same as those in Figure 1.

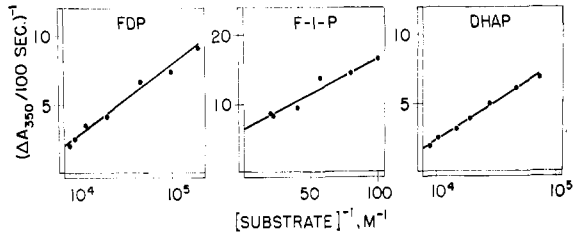

FIGURE 4: Double-reciprocal plots of rate of nitroform production *vs.* concentration of the substrates of aldolase. The data are taken from Figure 3 and the resulting numerical values of K_m' and V_{max}' are listed in Table III.

phosphate, or dihydroxyacetone. In addition, neither bovine serum albumin nor chymotrypsin accelerate TNM degradation in the presence of the aldolase substrates.

The rate of nitroform production in the presence of active aldolase and either DHAP or FDP is directly proportional to the concentration of enzyme (Figure 2). Aldolase first inactivated by treatment with TNM no longer induces nitroform production in the presence of substrates. Partially active aldolase increases the rate of nitroform production in direct proportion to its ability to cleave FDP (Table I). Strict dependence on the activity of aldolase is also apparent from the decrease in rate of nitroform production as a function of time (Figure 1) due to progressive inactivation of aldolase by TNM. Aldolase was inactivated to different degrees by borohydride reduction of the intermediate Schiff base formed in the presence of FDP (Horecker *et al.*, 1963). Again, the loss of activity toward FDP parallels the decrease in rate of nitroform production (Table II). Aldolase which has been completely inactivated by incubation in 4 M urea–0.05 M Tris-Cl–0.3 M NaCl (pH 8.2) for 30 min (Christen *et al.*, 1965) is no longer able to induce nitroform production upon addition of FDP (final concentration 1 mM) and 0.84 μmole of TNM.

The relationship between the rate of nitroform production and substrate concentration (Figure 3) suggests that the reaction depends on the formation of an enzyme–substrate complex. This is demonstrated by double-reciprocal plots of the velocity of nitroform production *vs.* substrate concentration which yield straight lines with all three substrates (Figure 4). The maximal rates of nitroform production (V_{max}') at saturating concentrations of substrate are listed in Table III together with the substrate concentrations resulting in half-maximal velocity (K_m'). The values of K_m' determined with different substrates are compared with the corresponding constants (K_m) obtained by enzymatic assay. K_m and K_m' are of the same order of magnitude for FDP and also for F-1-P. For DHAP, K_m was determined in the presence of GAP while K_m' was not, and hence these values may not be comparable directly.

Inorganic phosphate competitively inhibits the production of nitroform by aldolase in the presence of its substrate (Figure 5). The inhibition constant, K_i', is of the same order of magnitude as the reported value of K_i for the inhibition of FDP cleavage (Mehler, 1963).

TABLE II: Inactivation of Aldolase by Borohydride Reduction in the Presence of FDP. Concomitant Decrease in Rate of FDP Cleavage and of Nitroform Production in the Presence of Substrate.[a]

Inactivation by $NaBH_4$ + FDP		Control, $NaBH_4$ only	
FDP Cleavage (%)	Nitroform Production in the Presence of FDP (%)	FDP Cleavage (%)	Nitroform Production in the Presence of FDP (%)
54	54	91	94
29	29	85	85
16	18	92	96
8	10	87	83

[a] Aldolase was inactivated by the procedure of Horecker *et al.* (1963) using FDP instead of DHAP. The various degrees of inactivation were obtained by varying the pH (6–7) and the FDP concentration (0.2–2 mM) for the reduction with $NaBH_4$. Conditions for the reaction with TNM are as in Figure 1.

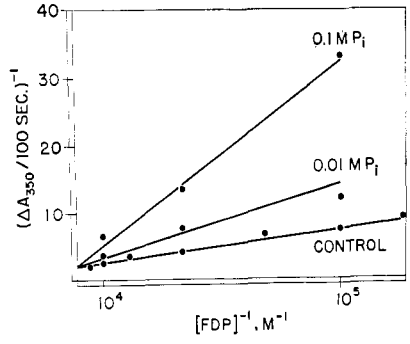

FIGURE 5: Competitive inhibition of nitroform production in the presence of aldolase and FDP by inorganic phosphate. Except for the phosphate, present at the indicated concentrations, conditions are as in Figure 1. K_i' is determined by the following (*cf.* Dixon and Webb, 1964):

$$K_i' = \frac{[P_i]}{(K_p/K_m) - 1} \cong 1.5 \times 10^{-2}\ M$$

TABLE III: V_{max}' and K_m' for Nitroform Production by Aldolase and Carboxypeptidase-Treated Aldolase in the Presence of Different Substrates. Comparison with V_{max} and K_m for the Enzymatic Cleavage–Condensation Reaction.[a]

Substrate	Native Aldolase (min^{-1})		Carboxypeptidase-Treated Aldolase (min^{-1})		Native Aldolase ($M \times 10^5$)		Carboxypeptidase-Treated Aldolase ($M \times 10^5$)	
	V_{max}'	V_{max}	V_{max}'	V_{max}	K_m'	K_m[b]	K_m'	K_m[c]
FDP	60	1900	200	91	3.0	6.1	5.4	2.0
F-1-P	19	76	43	95	1600	1200	2100	230
DHAP	71		160		3.6	210	32	

[a] Reaction conditions were as in Figure 1. The Lineweaver–Burk plots for native aldolase are shown in Figure 4 and for carboxypeptidase-treated aldolase in Figure 8. [b] Rutter *et al.* (1963). [c] Drechsler *et al.* (1959).

The rate of nitroform production at saturating concentrations of FDP depends on the concentration of TNM according to the equation $d[\text{nitroform}]/dt = k \times [\text{TNM}]^{0.68}$ (Figure 6). With DHAP as substrate an analogous dependency was found, the exponent being 0.57.

Experiments with limiting amounts of substrate demonstrate that substrate is consumed by the reaction with TNM in the presence of aldolase. Aldolase was added repetitively to a solution containing TNM and limiting amounts of DHAP. Immediately on addition of aldolase the rate of formation of nitroform increased both in the presence and absence of DHAP (Figure 7). However, in the presence of substrate the initial increase of the absorbance at 350 mμ was much greater than that for the control without substrate. The differences between the burst in nitroform production in the presence and absence of substrate no longer appeared after the third addition of aldolase, indicating that DHAP had been consumed in the TNM reaction. This was confirmed by enzymatic examination of the remaining DHAP concentration; 20 μl of mercaptoethanol was added to the reaction mixtures (*cf.* Figure 7) to reduce the remaining TNM. Addition of 0.4 ml of 2.56 mM NADH followed by 20 μg of α-glycerophosphate dehydrogenase to the aldolase–DHAP solution or to the DHAP–TNM solution yields the same decrease in absorbance at 340 mμ (0.10), demonstrating the presence of the initial amount of DHAP in both solutions. However, there was no decrease in absorbance at 340 mμ when this assay procedure was employed with the aldolase–DHAP–TNM solution. Analysis of the stoichiometry of the reaction, using limiting initial concentrations of DHAP or FDP, indicates that 3 moles of nitroform are produced during the aldolase-mediated reaction for every 2 moles of substrate consumed.

Rose *et al.* (1965) have demonstrated that treatment of aldolase with carboxypeptidase A apparently affects one distinct step in the reaction sequence of the enzyme. Therefore, K_m' and V_{max}' of carboxypeptidase-treated aldolase were determined, since these data might serve to identify the TNM-reactive enzyme–substrate intermediate. Again linear double-reciprocal plots were obtained (Figure 8) and the resulting kinetic constants are listed in Table III. For each substrate the rate of production of nitroform is higher with carboxypeptidase-treated aldolase than with native aldolase. For FDP the rate is tripled although the cleavage reaction is de-

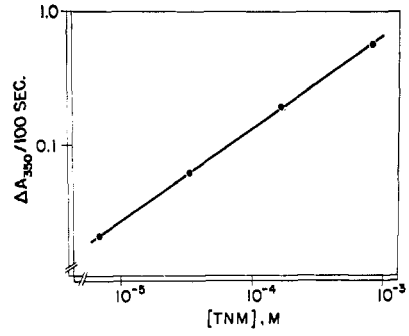

FIGURE 6: Rate of nitroform production by aldolase in the presence of FDP as a function of TNM concentration. The indicated final concentrations of TNM were obtained by addition of 25 μl of an appropriately diluted solution of TNM in 95% ethanol. Other conditions as in Figure 1.

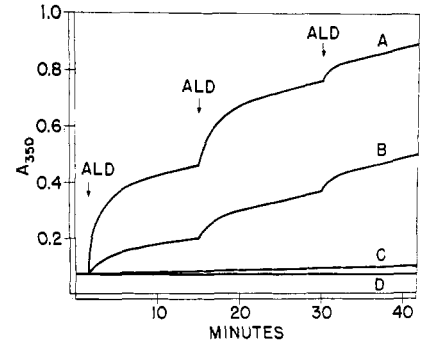

FIGURE 7: Consumption of the substrate in the aldolase-mediated reaction with TNM. The reaction mixture (A) contained 2.0×10^{-5} M DHAP and 2.1×10^{-4} M TNM; other conditions were as in Figure 1. At the indicated times 20 μl of aldolase solution (5.5 mg/ml) was added. The controls were kept under identical conditions except that they contained no DHAP (B), no aldolase (C), or no TNM (D), respectively. The difference in the amount of nitroform produced in A and B after 40 min together with the initial concentration of DHAP were employed to calculate the presumable reaction stoichiometry.

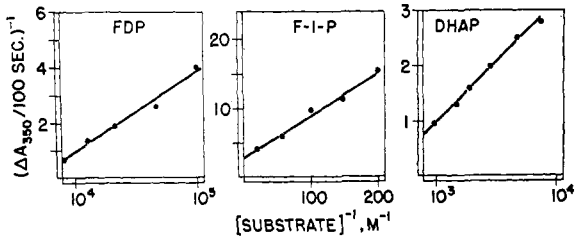

FIGURE 8: Carboxypeptidase-treated aldolase. Double-reciprocal plots of rate of nitroform production *vs.* concentration of the substrates. Carboxypeptidase-treated aldolase was prepared by incubating aldolase with carboxypeptidase A (115:1, w/w) in 0.05 M Tris-Cl–0.3 M NaCl (pH 8) for 1 hr at 0°. After that time the FDP activity had dropped to 5%, the F-1-P activity remaining virtually unchanged. The enzyme was used without separation from carboxypeptidase. The reaction mixtures contained the substrate at the indicated concentrations; the concentration of TNM and of enzyme and all other conditions as in Figure 1. The values of K_m' and V_{max}' are given in Table III.

creased to about 5%, for F-1-P it is doubled while the cleavage reaction is virtually unchanged, and for DHAP it is increased 2.3 times.

The differences in rate of nitroform production with FDP, F-1-P, or DHAP as substrates and the results with carboxypeptidase-treated aldolase suggest that the TNM-reactive intermediate is the DHAP carbanion of the enzyme–substrate complex (see Discussion section). Nonenzymic model systems containing stable carbanions were therefore tested in regard to their reactivity toward TNM.

The production of nitroform in alkali is markedly enhanced by acetone (Figure 9) which under these conditions exists in equilibrium with its mesomeric enolate carbanion (*cf.* Gould, 1959). At neutral pH acetone does not enhance nitroform production. Isopropenyl acetate which contains a double bond in the same position as the enolate of acetone does not react with TNM, thus excluding a reaction of this double bond with TNM under the present conditions.

In the pyridoxal-catalyzed reactions of glutamic acid a metal-stabilized carbanion is postulated as an intermediate (Braunstein and Shemyakin, 1953; Metzler

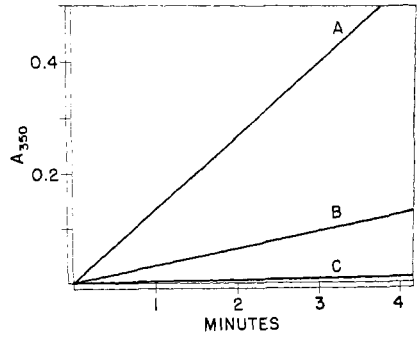

FIGURE 9: Production of nitroform in the presence of acetone at alkaline pH. The reactions were carried out in 1-cm spectrophotometer cuvets at 25° by addition of 50 μl of 0.0336 M TNM to (A) 100 μl of acetone and 2.85 ml of 0.09 M ammonium chloride (pH 10.0); (B) 2.95 ml of 0.09 M ammonium chloride (pH 10.0); and (C) 100 μl of acetone and 2.85 ml of 0.05 M Tris-Cl–0.3 M NaCl (pH 7.0).

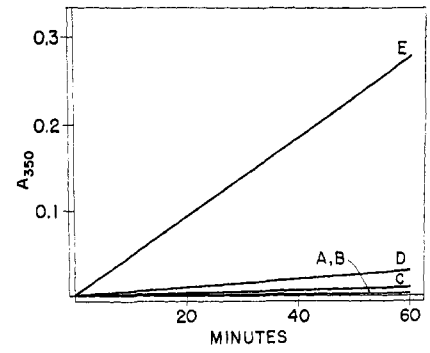

FIGURE 10: Production of nitroform in the presence of pyridoxal, glutamic acid, and aluminum ions. The reactions were carried out in spectrophotometer cuvets at 25° by the addition of 100 μl of 0.0336 M TNM to 2 ml of 0.1 M sodium acetate (pH 5.0) containing: (A) 0.001 M aluminum sulfate; (B) 0.001 M glutamic acid and 0.001 M aluminum sulfate; (C) 0.001 M pyridoxal hydrochloride and 0.001 M aluminum sulfate; (D) 0.001 M glutamic acid and 0.001 M pyridoxal hydrochloride; and (E) 0.001 M glutamic acid, 0.001 M pyridoxal hydrochloride, and 0.001 M aluminum sulfate. Solutions without TNM but otherwise identical composition served as spectrophotometer blanks.

et al., 1954). Nitroform is rapidly released from TNM in the presence of glutamic acid, pyridoxal, and aluminum ions (Figure 10), but without aluminum ions the reaction is very slow. When glutamic acid or pyridoxal alone is present with TNM practically no nitroform is produced. Enhanced formation of nitroform was also observed when aluminum ions were added to a mixture of TNM, α-ketoglutaric acid, and pyridoxamine.

Again, thiamine, which has carbanionic character at pH 8.0 (Breslow, 1957), reacts readily with TNM as judged by the increase in the absorbance at 350 mμ (Figure 11). The reaction is first order with respect to thiamine. With limited amounts of thiamine in the presence of excess TNM the reaction slows down with time, suggesting that the thiamine is consumed by the reaction.

Discussion

While TNM has proven valuable for modifying tyrosyl and sulfhydryl groups of proteins the present data

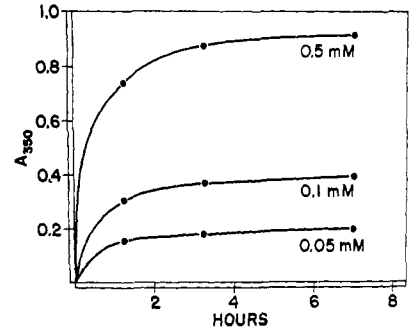

FIGURE 11: Production of nitroform in the presence of thiamine. The reaction mixture contained thiamine in the indicated concentrations in 2 ml of 0.05 M Tris-Cl (pH 8.0), room temperature. At zero time 0.5 ml of 0.0336 M TNM was added and at the indicated times aliquots were diluted tenfold for determination of A_{350}. Readings were corrected for the production of nitroform in the absence of thiamine.

demonstrate its usefulness in reacting specifically with an enzyme–substrate intermediate. The production of nitroform from TNM in the presence of aldolase and its substrates clearly differs from the nonspecific breakdown of TNM previously observed with a number of proteins including aldolase. The latter has been attributed to catalysis by protein side-chain groups (Sokolovsky *et al.*, 1966; Riordan and Christen, 1968). In the present instance, the marked over-production of nitroform occurs only when both aldolase *and* substrate are present in the reaction mixture, and only when the enzyme is active. Moreover, and most important, only substrates for the enzymatic cleavage–condensation reaction enhance nitroform production (Figure 1). Since a reaction of TNM either with the products of the aldolase cleavage reaction, GAP and DHAP, or with aldolase substrates in the presence of proteins other than aldolase was excluded, the enhanced nitroform production seemed to be due to a specific interaction of an aldolase–substrate complex with TNM.

In the presence of aldolase and substrate, the rate of nitroform production is proportional to the concentration of active enzyme, as shown in experiments with varying amounts of native aldolase (Figure 2) or in experiments with partially inactivated aldolase (Tables I and II). Both in the TNM-inactivated enzyme (Table I) as well as in that inactivated by borohydride reduction of the Schiff base intermediate (Table II), the decrease of nitroform production in the presence of substrate parallels the loss of cleavage activity. Similarly, aldolase which has been completely inactivated by incubation in 4 M urea no longer induces enhanced nitroform production in the presence of substrates. The proportionality between the rate of nitroform production and residual enzymatic activity of partially inactivated aldolase suggests that the TNM degradation is directly dependent on the enzymatic reaction itself.

The linear double-reciprocal plots of rate of nitroform production *vs.* substrate concentration (Figure 4) demonstrate that saturation kinetics with respect to substrate also apply for the reaction with TNM. Furthermore, with FDP and F-1-P as substrates, the K_m' (the substrate concentration at which nitroform production occurs at half-maximal rate) corresponds closely with the K_m of the usual enzymatic reactions (Table III). These results indicate that TNM reacts with an aldolase–substrate complex, specifically with the same complex that promotes the cleavage reaction.

The differences in K_m' and K_m for DHAP may be attributed to the different conditions employed. The K_m for the condensation reaction was determined in the presence of GAP which was absent during the reaction with TNM.

Inorganic phosphate competitively inhibits the degradation of TNM in the presence of aldolase and substrate, the K_i' (Figure 5) being of the same order of magnitude as the K_i for the FDP-cleavage reaction (Mehler, 1963), yielding further evidence for a reaction between TNM and an enzyme–substrate intermediate.

The stoichiometry (amount of substrate consumed is two-thirds of nitroform produced; *cf* Figure 7) and the dependence of the rate of nitroform production on the concentration of TNM (Figure 6) cannot be interpreted further on the basis of the data presently available.

According to the reaction sequence proposed by Rose *et al.* (1965) and the formation of a Schiff base between aldolase and substrate (Horecker *et al.*, 1963; Rose and O'Connell, 1967), the reaction mechanism of aldolase may be presumed to proceed as in eq 1.

$$\text{aldolase} + \text{FDP (F-1-P)} \rightleftharpoons \underset{\substack{\text{carbanion of}\\ \text{Schiff base}\\ +\\ \text{GAP (GA)}}}{\text{aldolase–DHAP}^-} \overset{+H^+}{\underset{-H^+}{\rightleftharpoons}}$$

$$\underset{\text{Schiff base}}{\text{aldolase–DHAP}} \rightleftharpoons \text{aldolase} + \text{DHAP}^- \qquad (1)$$

Since the reaction of TNM with an enzyme–substrate intermediate occurs with FDP and F-1-P as well as with DHAP, the TNM-reactive intermediate must be located in a segment of the reaction sequence common to all of these substrates, *i.e.*, between the carbanion of the DHAP–aldolase Schiff base and the liberation of DHAP from the enzyme. Thus, the reaction with TNM occurs after the cleavage of the hexose phosphates, and the TNM-reactive intermediate would be identical whether FDP or F-1-P is employed as substrate. Therefore, in the steady state of the cleavage reaction of either substrate the rate of nitroform production would be a measure of the relative concentration of this reactive intermediate. The V_{max}' for nitroform production with F-1-P as substrate is about one-third that obtained with FDP (Table III), indicating that the TNM-reactive intermediate is located after the rate-limiting step for the cleavage of F-1-P. As demonstrated by Rose *et al.* (1965), this step occurs prior to the formation of the C–H bond of DHAP.

Treatment of muscle aldolase with carboxypeptidase A apparently affects the formation of the C–H bond of DHAP, lowering the rate of this step such that it becomes rate limiting in both FDP and F-1-P cleavage (Rose *et al.*, 1965). The rate of nitroform production induced by carboxypeptidase-treated aldolase with FDP as substrate is three times higher than that of native aldolase, while the cleavage reaction is only 5% of that of the native enzyme (Table III). With F-1-P as substrate the rate of nitroform production is doubled while the cleavage reaction remains unchanged. Thus, it would appear that the TNM-reactive intermediate is located prior to the step affected by the carboxypeptidase treatment, the amount of TNM-reactive intermediate which accumulates being greater in the steady state of the cleavage reaction catalyzed by carboxypeptidase-treated aldolase than in that catalyzed by the native enzyme.

Taken together these data suggest that the carbanion of the DHAP–aldolase complex is the species which reacts with TNM. This intermediate has all of the properties predicted on the basis of the above experiments. It is located after the rate-limiting step of F-1-P cleavage, it is formed when DHAP is the substrate, and it is located prior to the step affected by carboxypep-

tidase. Alternatively, TNM might be thought to react with an enamine resonance form of the carbanion. The negative results obtained with isopropenyl acetate make this unlikely, however.

The finding that the rate of the reaction between TNM and the enzyme–substrate complex of carboxypeptidase-treated aldolase is also enhanced when DHAP serves as substrate suggests that the effect of carboxypeptidase digestion may not be restricted entirely to the C–H bond-forming step. With FDP and F-1-P as substrate the reaction with TNM occurs under steady-state conditions so that a decrease in the rate of the C–H bond-forming step will lead to an accumulation of the carbanion intermediate. In contrast, with DHAP as substrate an equilibrium is established which would not be shifted by decreasing the rates of the C–H bond-forming and -breaking steps alone. Hence, the structure of the carbanion intermediate itself has to be altered either in the sense of an enhanced reactivity toward TNM or of a shift in the equilibrium in its favor.

The turnover numbers for nitroform production which are much greater than 1 min^{-1} for all substrates (Table III) indicate that the enzyme itself is not inactivated by the reaction between TNM and the enzyme–substrate intermediate. A relatively slow inactivation of aldolase occurs (Figure 1) which is independent of this reaction but rather is due to a nonspecific oxidation of sulfhydryl groups of the enzyme by TNM (Riordan and Christen, 1968).

On the other hand, the substrate *is* consumed by the reaction of TNM with the enzyme–substrate intermediate (Figure 7), indicating that TNM reacts irreversibly with the DHAP moiety of the aldolase–DHAP carbanion. TNM is thought to have partial ionic character which facilitates the production of nitronium ions which in turn would react as proton analogs with the DHAP carbanion. Thus, the most probable scheme for the reaction between TNM and the aldolase–substrate intermediate seems to be a reaction between the DHAP carbanion and TNM leading to a derivative of DHAP. Attempts at isolation and identification of this derivative have not been successful thus far.

The behavior of TNM toward several nonenzymatic model systems in which a carbanion intermediate is known to be present is similar and supports such a scheme. Thus, the mechanism of the reaction of TNM and acetone in alkali (Figure 9) is perhaps analogous to that for the halogenation of acetone in alkali. The latter has been shown to involve a mesomeric carbanion–enolate intermediate (*cf.* Gould, 1959). Pyridoxal in the presence of suitable metal ions has been shown to catalyze reactions of amino acids in a fashion analogous to the pyridoxal phosphate enzymes (Braunstein and Shemyakin, 1953; Metzler *et al.*, 1954). The reactions involve an intermediary carbanion of the α-carbon atom of the amino acid. The breakdown of TNM in the presence of pyridoxal and glutamic acid is greatly accelerated by aluminum ions (Figure 10). Similarly, as judged by nitroform production, thiamine, which has a stable carbanion at C-2 of the thiazolium ring (Breslow, 1957), reacts readily with TNM at pH 8.0 (Figure 11). The reactivity of TNM with various compounds, only under conditions where these are known to produce carbanions, strongly supports the view of a carbanion-specific reactivity of TNM.

Previously we have reported that the substrate protects aldolase against inactivation by TNM (Riordan and Christen, 1968). The present findings demonstrate that in addition to specifically blocking essential groups this protection must include a continuous decrease in the concentration of TNM by reaction with the aldolase–substrate intermediate. This possibility of a reaction between the modifying reagent and an enzyme–substrate intermediate is not generally appreciated and should be borne in mind when performing and interpreting protection experiments. In instances when the product is not readily detected, as in the reaction of *N*-ethylmaleimide with an enzyme–substrate intermediate involving the cystathionine cleavage enzyme of *Neurospora* (Flavin and Slaughter, 1964; Flavin, 1965), such a reaction might remain undetected.

Several characteristics make TNM appear a useful reagent for chemical modification of enzyme–substrate intermediates: (1) it exhibits a specific reactivity toward carbanions; (2) its reaction can be detected easily and followed directly by virtue of the strong absorbance of the nitroform produced; (3) in most instances it may react irreversibly as in the case of aldolase and its substrates and with thiamine, thus allowing covalent labeling of the intermediary nucleophilic carbon atom; (4) its reaction is slow, *i.e.*, at appropriate concentrations of TNM only a small percentage of all molecules passing through the reactive intermediate state will react with TNM (Table III). Hence, the TNM–carbanion reaction may not disturb the steady state of the system appreciably and the rate of nitroform production may provide a convenient gauge of the relative concentration of the carbanion intermediate under different conditions.

Though the unique reactivity toward specific reagents might be a property of activated intermediates in general, the value of modification of enzyme–substrate complexes as a general approach to the study of the reaction mechanism of enzymes has not been emphasized. As illustrated with TNM and aldolase, chemical modification of an enzyme–substrate complex with a proper specific reagent will be useful not only in detection and identification of an intermediate but also for detailed kinetic study of the reaction sequence of the enzyme.

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The Inhibition of Acetylcholinesterases by Anionic Organophosphorus Compounds*

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ABSTRACT: Data on seven pairs of organophosphates confirm the view that O desalkylation normally reduces anticholinesterase activity profoundly; the average reduction was 158,000-fold. By contrast, O desalkylation of certain tertiary amine-containing organophosphates only reduces activity by 60- to 292-fold. Thermodynamic and kinetic studies on the inhibition process, coupled with analog synthesis and studies on pK_a, on the nature of the inhibited enzyme, and on joint inhibition of cholinesterase by selected pairs of organophosphates, suggest that internal salt formation can reduce the anionicity of certain desalkylated organophosphates, and permit them to be effective anticholinesterases.

It is well established that the organophosphates inhibit AChE[1] by phosphorylating its esteratic site (Wilson, 1951; Aldridge and Davison, 1953; O'Brien, 1960). Recently the role of complex formation prior to phosphorylation has been indicated and evaluated (Main, 1964; Main and Hastings, 1966; Main and Iverson, 1966). The terminology of eq 1 will be employed, where P is the dialkyl phosphoryl group, X the leaving group, and E the enzyme. Main (1964) has shown that

$$E + PX \underset{}{\overset{K_a}{\rightleftharpoons}} EPX \xrightarrow{k_2} EP \xrightarrow{k_3} E + P \qquad (1)$$

the apparent bimolecular rate constant as normally measured, k_i, is equal to k_2/K_a, and would better be called the bimolecular reaction constant. The k_3 (reactivation) step is slow enough to be ignored in most cases, except where reactivators are employed.

Because the phosphorylation step involves an electrophilic attack by the phosphorus, its rate is promoted by electrophilic substituents attached to the phosphorus. For the same reason, hydrolytic removal of one of the ester groups of these phosphotriesters profoundly reduces anticholinesterase activity, because it has the effect of attaching an anion to the phosphorus, for the POH group exists virtually entirely as PO^- at physiological pH. This fact, though very poorly documented, is the basis of the general view that such a hydrolysis (for instance, in metabolism of these compounds) constitutes virtually a total detoxication. We were therefore surprised to note that compounds such as $(C_2H_5O)(HO)P(O)SCH_2CH_2N^+(C_2H_5)_2(CH_3)$ had "unusual effectiveness" as insecticides (Snyder, 1960). Similarly, Heilbronn-Wikström (1965) reported good anticholin-

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[1] Abbreviations used in this paper that are not defined in *Biochemistry 5*, 1445 (1966), are: AChE, acetylcholinesterase; 2-PAM, 2-pyridinealdoxime methiodide.