Suicide Inactivation of Fructose-1,6-bisphosphate Aldolase†

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ABSTRACT: 2-Keto-4,4,4-trifluorobutyl phosphate (HTFP) was prepared from 3,3,3-trifluoropropionic acid. HTFP acts as an irreversible inhibitor of rabbit muscle aldolase: the loss of activity was time dependent and the inactivation followed a pseudo-first-order process. Values of 1.4 mM for the dissociation constant and 2.3×10^{-2} s^{-1} for the reaction rate constant were determined. The kinetic constants do not depend on the enzyme concentration. No effect of thiols on the inactivation rate was detected. Only 1–2 mol of fluoride ions was liberated per inactivated subunit, indicative of a low partition ratio. Dihydroxyacetone phosphate protected the enzyme against the inactivation in a competitive manner, and glyceraldehyde 3-phosphate protected as if it formed a condensation product with HTPF. 5,5′-Dithiobis(2-nitrobenzoic acid) thiol titration showed the loss of one very reactive thiol group per enzyme subunit after inactivation. All those observations seem to agree with a suicide substrate inactivation of aldolase by HTPF.

The study of suicide substrates constitutes a growing area of interest for scientists involved in the field of both chemistry and biochemistry (Bloch, 1969; Walsh, 1981; Seiler et al., 1978).

The interest of inhibition by suicide substrates lies in their specificity toward a target enzyme. Those compounds are natural substrate analogues containing a latent functional group that is uncovered only after binding and reaction with the enzyme. The activated molecule then reacts with the enzyme, leading to irreversible enzyme inactivation (Abeles & Maycock, 1976). This type of inactivator is of interest in producing therapeutic agents, antiparasites, insecticides, and plant controls agents.

In this paper, we shall report the synthesis of an analogue of DHAP,[1] 2-keto-4,4,4-trifluorobutyl phosphate (HTFP), and the inactivation of rabbit muscle fructose-1,6-bisphosphate aldolase (Horecker et al., 1972) by this reagent. This aldolase has been shown to catalyze the hydrogen exchange of acetol phosphate with solvent (Rose & O'Connell, 1969; Pratt, 1977) and to be inactivated by 2-Keto-3-butenyl phosphate (Motiu-De Grood et al., 1979). So it was worthwhile to prepare HTFP and to study its inhibitory action on aldolase.

Affinity labeling of aldolase with *N*-(bromoacetyl)-ethanolamine phosphate has been shown to involve His-359 at pH 6.5 and Lys-146 at pH 8.5 (Hartman et al., 1973; Hartman & Welde, 1974; Hartman & Brown, 1976). Lys-146 may have been labeled during a paracatalytic modification (Christen, 1979). Arg-55 was implied in the binding site of the phosphate group at C-1 of fructose 1,6-bisphosphate (Patthy et al., 1979). The thiol reactivity and the effects of their modification have been extensively studied and will be discussed later in the perspective of the results presented here.

Materials and Methods

Enzymes. The fructose-1,6-bisphosphate aldolase from rabbit muscle and the triosephosphate isomerase/glycerol-1-phosphate dehydrogenase (1:10) from rabbit muscle, both as a suspension in 3.2. M ammonium sulfate solution, were purchased from Boehringer (Mannheim). Before use, the suspensions were centrifuged, and the pellet was dissolved in a minimum of stock buffer and dialyzed overnight against the same buffer. The aldolase had a specific activity of 15–18 units/mg.

Reagents. DHAP as dimethyl ketal, FDP, GSH, and NADH were obtained from Boehringer (Mannheim). GAP as diethyl ketal, DTNB, and DTT were purchased from Sigma Chemical Co. Ethyl 3,3,3-trifluoropropionate was prepared by Dr. Lantz (ATOCHEM, Lyon), hydrolyzed to the acid (Peters et al., 1971), and transformed to the corresponding acid chloride by action of phosphorus pentachloride (Henne et al., 1955).

Synthesis. (*a*) *1-Diazo-2-keto-4,4,4-trifluorobutane.* A solution of 3,3,3-trifluoropropionyl chloride (2.1 g; 14 mmol) in anhydrous ether (50 mL) was added dropwise at 0 °C to a 0.3 M diazomethane etheral solution (190 mL) dried over potassium hydroxide. After 18 h at 20 °C, excess diazomethane and ether were evaporated, and the yellow liquid obtained was purified by chromatography on silica gel (hexane–ether, 30/70). The yield was 54% (1.2 g): ^{1}H NMR ($CDCl_3$, tetramethylsilane) δ 5.48 (s, 1 H), 3.13 (q, 2 H, J = 9 Hz); IR ($CHCl_3$) 2120, 1645 cm^{-1}.

(*b*) *HTFP.* A solution of the diazo ketone prepared as in (a) (0.16 g, 1 mmol) and 85% phosphoric acid (0.7 mL) in ether (15 mL) was stirred at 20 °C for 60 h. The product was extracted with water (twice with 10 mL). The waters layers were washed twice with ether (10 mL) and concentrated under vacuum. Excess phosphoric acid was removed by ion-exchange chromatography on Dowex 1 (elution gradient 40–100 mM HCl). After evaporation under reduced pressure at 25 °C, a white powder was obtained in a yield of 50%: ^{1}H NMR (CD_3COCD_3, tetramethylsilane) δ 3.33 (q, 2 H), 4.33 (d, 2 H), 10.3 (s, 2 H). Anal. Calcd for $C_4H_6O_5F_3P$: C, 21.63; H, 2.72; P, 13.94. Found: C, 21.50; H, 2.72; P, 13.86.

Assay Method. Aldolase activity was assayed by means of a modification of the usual triosephosphate isomerase/glycerol-1-phosphate dehydrogenase method (Rajkumar et al., 1966). Assays were carried out in 100 mM TEA–HCl buffer, pH 7.5, at 30 °C. The conditions for a typical experiment were the following: 70 μM NADH, 0.5 mM FDP, triosephosphate isomerase/glycerol-1-phosphate dehydrogenase (1:10) at about

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[1] Abbreviations: DHAP, dihydroxyacetone phosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; FDP, fructose 1,6-bisphosphate; GAP, glyceraldehyde 3-phosphate; GSH, glutathione; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HTFP, 2-keto-4,4,4-trifluorobutyl phosphate (hydroxyketotrifluorobutane monoester of phosphoric acid); NADH, reduced nicotinamide adenine dinucleotide; TEA, triethanolamine.

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Scheme I

$$CF_3CH_2COCl \xrightarrow{CH_2N_2} CF_3CH_2COCHN_2 \xrightarrow{H_3PO_4} CF_3CH_2COCH_2OPO_3H_2$$

20 μg, and aldolase at 2×10^{-3} μM (expressed as subunits). Activity was monitored by measuring the decrease of the absorption at 340 nm on a Cary 118 spectrophotometer. Aldolase concentration was determined by measuring the absorption at 280 nm with $\epsilon_{1cm}^{0.1\%} = 0.93$ (Donovan, 1964).

Inactivation Studies. The inactivation of aldolase (3.5 μM active subunits) by HTFP (0.1–2 mM) was performed in 100 mM TEA–HCl buffer, pH 7.5, at 30 °C. At given times, aliquots (4 μL) were withdrawn and diluted in 1 mL of assay solution. Time-dependent loss of activity was determined as above. Under the same conditions, the influence of GSH (1 mM) and DTT (1 mM) on the inactivation rate was determined at 0.84 mM HTFP.

In order to exclude that a decomposition product from HTFP is the inactivator, HTFP (1 mM) was incubated at 30 °C in 100 mM TEA–HCl buffer, pH 7.5. At given times (0, 10, and 30 min), aldolase was added to an aliquot, and the inactivation rate was followed as above.

Dependence of Inactivation Rate on Enzyme Concentration. In 100 mM TEA–HCl buffer, pH 7.5, at 30 °C, the inactivation rate of aldolase (6 and 60 μM active subunits) by HTFP (4.2×10^{-4} M) was determined as above.

Protection against Inactivation by DHAP and GAP. Under the same conditions of inactivation, the concentration of DHAP (28–140 μM) and of GAP (35–350 μM) was varied at a 1 mM HTFP concentration. Further study of the effect of GAP on the inactivation was done at HTFP concentrations of 0.21, 0.265, 0.84, and 1 mM and at GAP concentrations of 90 and 180 μM.

Thiol Determination (Riddles et al., 1983). The reaction of enzyme with DTNB (0.46 mM) in 100 mM phosphate buffer, pH 8, was followed by measuring the increase of the absorption at 412 nm, with an extinction coefficient of 13 600 M^{-1} cm^{-1} for the 5-thio-2-nitrobenzoate anion (Ellman, 1959). The protein to be titrated was dialyzed exhaustively against buffer under oxygen-free conditions. All solutions and buffers were bubbled with argon before use. After reaction of the enzyme with HTFP, the excess of reagent was eliminated by dialysis against 100 mM phosphate buffer, pH 8.

Fluoride Determination. Fluoride ion concentration was determined with a Metrohm ionometer 610 equipped with a fluoride electrode EA 306-F and a reference cell. The solution was slowly stirred with a magnetic stirbar. The decrease in potential was recorded as a function of time and compared with a calibration curve from known concentrations of fluoride ions.

Results

Stability of Inactivator. The synthesis of HTFP was performed as shown in Scheme I in satisfactory yield. The analytical data agreed with the proposed structures. The stability of HTFP was tested with the fluoride-specific electrode at pH 7.5 in 100 mM TEA–HCl buffer and at pH 7 in 100 mM HEPES buffer, at 30 °C. The rates of fluoride ion appearance are given in Table I. At these pH values, the stability of HTFP is sufficient to perform inactivation tests. Under these experimental conditions, the final value of fluoride ion concentration never reached the expected value of three fluoride ions/mol of HTFP. This value was only obtained after basic treatment (pH 9).

The concentration of fluoride ion liberated was determined during the inactivation of aldolase. A value of one to two F^- liberated by inactivated enzyme subunit could be calculated.

Table I: Fluoride Ion Appearance as a Function of Time from HTFP (1 mM) Addition

time (min)	concn of fluoride ion (mM): 100 mM TEA–HCl, pH 7.5	100 mM HEPES, pH 7
10	0.455	0.28
20	0.84	0.56
30	1.1	0.82
60	1.75	1.45
120	2.30	2.0
240	2.50	2.4
360	2.60	2.60
after treatment at pH 9	3.00	3.00

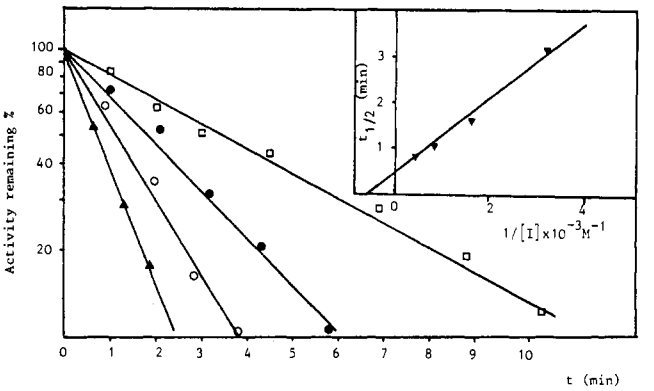


FIGURE 1: Inhibition of aldolase by HTFP. Assays were carried out in the presence of HTFP at 1.6 (▲), 0.84 (○), 0.42 (●), or 0.21 mM (□). Other experimental conditions were as described under Materials and Methods.

Kinetics of Inactivation. Loss of enzyme activity in the presence of a suicide substrate is time dependent and obeys a pseudo-first-order process. The kinetic equation for the suicide inactivation is similar to that established by Kitz & Wilson (1962) for affinity labeling:

$$t_{1/2} = \frac{\log 2}{k_{cat}}\left(1 + \frac{K_I}{[I]}\right)$$

where [I] is the inhibitor concentration, K_I is the apparent dissociation constant, and k_{cat} is the transformation rate of the inhibitor by the enzyme. In both mechanisms, affinity labeling and suicide inactivation, one observes first a reversible binding of the molecule to the enzyme, followed by an irreversible step.

For aldolase and HTFP, the inactivation kinetics correspond to a pseudo-first-order process (Figure 1). Saturation kinetics are observed with a $K_I = 1.4$ mM and a k_{cat} of 2.3×10^{-2} s^{-1}.

However, eq 1 describes an ideal case where every activated

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} EI \xrightarrow{k_{cat}} EI' \xrightarrow{k_{inact}} E_{inact} \qquad (1)$$

form I′ leads to the inactivation. But some release of I′ can occur; the dissociation and the inactivation are thus competing reactions.

Since the activated form I′ is generally a reactive molecule, it tends to react with any reagent (X) present in solution to give I′–X, a nonreacting species (eq 2). A partition ratio has

$$\begin{array}{l} E + I \rightleftharpoons EI \longrightarrow EI' \longrightarrow E_{inact} \\ \qquad\qquad\quad \downarrow \\ \qquad\qquad\quad E + I' \\ I' + X \longrightarrow I'{-}X \end{array} \qquad (2)$$

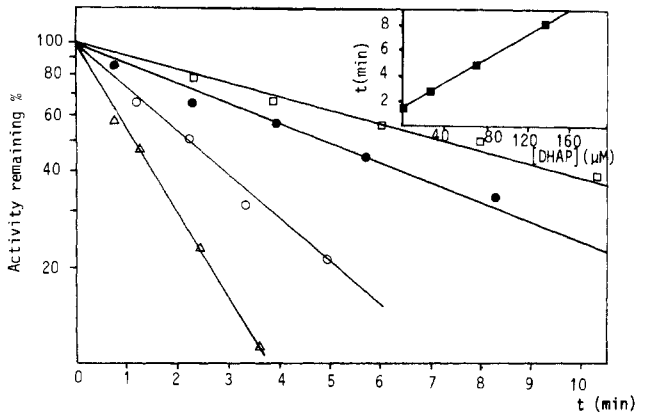


FIGURE 2: Protection against inactivation by DHAP. Assays were carried out in the presence of 1 mM HTFP. The concentrations of DHAP were 0 (△), 28 (○), 70 (●), and 140 μM (□). Other experimental conditions were as described under Materials and Methods. (Insert) Plot of $t_{1/2}$ vs. [DHAP].

been defined as the number of product molecules released to the number of inactivated enzyme molecules (Walsh, 1981).

If I′ reacts with the enzyme back from solution, two mechanisms can occur: a second-order process (eq 3) or an

$$E + I \rightleftharpoons EI \rightarrow EI' \rightarrow E + I' \rightarrow E_{inact} \qquad (3)$$

affinity labeling (eq 4). For eq 4, inactivation kinetics show

$$\begin{array}{ccccc} E + I & \rightleftharpoons & EI & \longrightarrow & EI' \longrightarrow E_{inact} \\ & & \downarrow\uparrow & & \\ & & E + I' & & \end{array} \qquad (4)$$

a lag period followed by an increasing inactivation rate. These latter cases (eq 3 and 4) may be detected by addition of a species reacting with I′ during the course of inactivation. The result will be a decrease in the inactivation rate. Another possibility for testing this mechanism consists of making a second addition of enzyme to the incubation mixture containing enzyme inactivated by an excess of inhibitor. If the inactivation rate is the same as that for the first one, it will confirm that the inactivation is not due to an accumulating material (Abeles & Maycock, 1976). Another test is that the inactivation kinetic constants K_I and k_{cat} should not be enzyme-concentration dependent. If a second-order inactivation (eq 3) or an affinity labeling (eq 4) is really causing the activity loss at constant suicide substrate concentration, an increase of the enzyme concentration could lead to an increased production of I′ and then to an increase of the inactivation rate above that calculated for a suicide inhibition (eq 1).

In order to exclude the inactivation of aldolase by a product released and accumulated in the solution, different experiments were carried out. Addition of GSH or DTT (1 mM) did not change the inactivation rate. After preincubation of HTFP in the assay buffer, the added aldolase showed a lower time-dependent inactivation rate (results not shown). After 75% of the enzyme was inactivated by an excess of HTFP, a second addition of reactive aldolase was made. The inactivation rate was reduced (results not shown). Finally, the inactivation kinetic constants did not depend on the enzyme concentration. The fact that 1–2 mol of fluoride ions was released per inactivated enzyme subunit indicates that the partition ratio is small (≤1).

After inactivation, the enzyme was exhaustively dialyzed against TEA–HCl buffer, and no activity was restored. The inactivation is irreversible.

Protection against Inactivation by DHAP and GAP. The addition of DHAP or GAP in the incubation mixture reduced

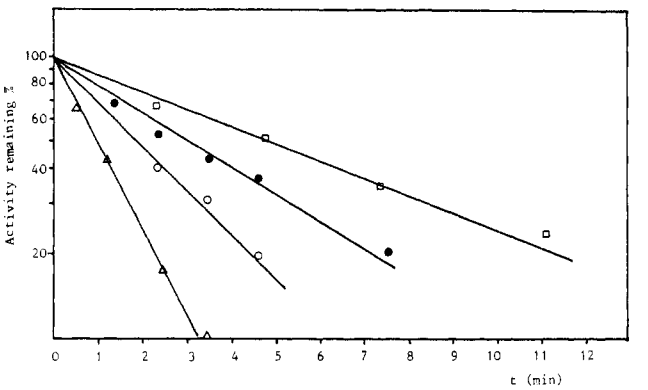
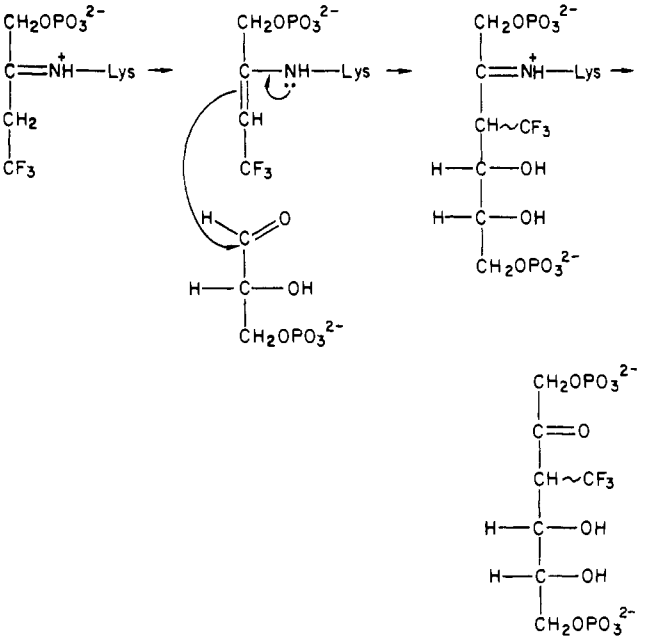


FIGURE 3: Protection against inactivation by GAP. Assays were carried out in the presence of 1 mM HTFP. The concentrations of GAP were 0 (△), 35 (○), 89 (●), and 350 μM (□). Other experimental conditions were as described under Materials and Methods.

Scheme II



the rate of inactivation of aldolase by HTFP (Figures 2 and 3). For DHAP, the kinetic data were treated according to the equation established by Meloche, implying that the suicide substrate and DHAP compete for the same binding site (Meloche, 1967). The dissociation constant for DHAP calculated from Figure 2 was 18 μM, which is close to the value of 16 μM found by fluorescence quenching (Rose & O'Connell, 1969) and higher than the value of 1.4 μM determined by the inhibition of aldolase inactivation with 2-keto-3-butenylphosphate (Motiu-De Grood et al., 1979).

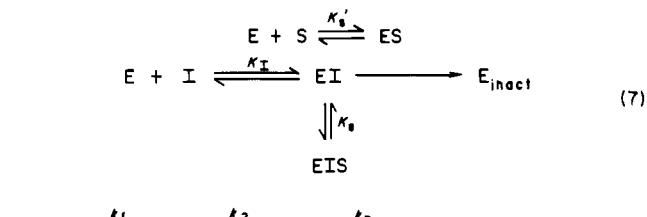
The inhibition of the inactivation by addition of GAP (S) was studied in order to determine if GAP only hinders the inactivation by binding to the enzyme and/or if it condenses with HTFP (I) bound to aldolase, giving 3-(trifluoromethyl)-3-deoxyfructose 1,6-bisphosphate (P) (Scheme II). This could be described by eq 5, and the deduced inactivation

$$\begin{array}{ccccc} E + I & \overset{K_I}{\rightleftharpoons} & EI & \xrightarrow{k} & E_{inact} \\ & & + & & \\ & & S & & \\ & & \downarrow\uparrow K_s & & \\ & & EIS & & \end{array} \qquad (5)$$

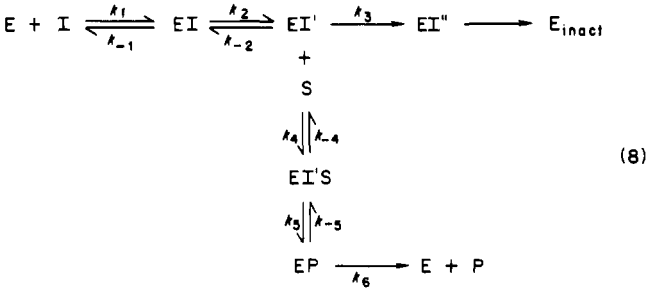
kinetic should correspond to eq 6. Rate law 6 was established

$$t_{1/2} = \frac{\log 2}{k}\left(1 + \frac{K_I}{[I]} + \frac{[S]}{K_s}\right) \quad (6)$$

with the assumption that formation of the complexes EI and EIS is under thermodynamic control. When $t_{1/2}$ is plotted as a function of [S] for differing concentrations of I, the slope of the line should be independent of [I]. In fact, for increasing concentration of I, a set of nonparallel lines is obtained (Figure 4). This could be explained by more complex mechanisms 7 and 8. In mechanism 7, we consider that GAP could bind



(7)



(8)

to the DHAP binding site (Mehler, 1963; Rose & O'Connell, 1969). In mechanism 8, the condensation of the enolic form of HTFP with GAP gives rise to the diphosphate P, whose cleavage by the enzyme is considered negligible. The rate laws 9 and 10 were deduced for mechanisms 7 and 8, respectively.

$$t_{1/2} = \frac{\log 2}{k}\left[1 + \frac{K_I}{[I]} + \left(\frac{1}{K_s} + \frac{1}{K_s'}\frac{K_s}{[I]}\right)[S]\right] \quad (9)$$

$$t_{1/2} = \frac{\log 2}{k_3}\left[\alpha + \frac{\beta}{[I]} + \left(\gamma + \frac{\delta}{[I]}\right)[S]\right] \quad (10)$$

α, β, γ, and δ are constants depending on the rate constant k_1, k_{-1}, ..., k_5, k_{-5}.

According to both eq 9 and 10, the slope should depend on the concentration of HTFP. In a preliminary trial, a product that chromatographed on Dowex 1 like FDP and that liberated fluoride ions on basic treatment was isolated in a 10% yield and was not detected in an assay without enzyme. Further studies are required in order to ascertain its structure: 3-(trifluoromethyl)-3-deoxyfructose 1,6-bisphosphate.

Thiol Determination. One thiol per subunit was titrated with DTNB in less than 30 s on the native aldolase. After reaction with HTFP, this more reactive thiol has disappeared.

Discussion

The suicide inactivation of aldolase by HTFP is indicated by pseudo-first-order kinetics, by the lack of any effect of thiols on the inactivation rate, by the invariability of the inactivation kinetic constants on the enzyme concentration, and by a decrease of this rate after addition of enzyme to an incubation mixture containing enzyme inactivated up to 75% by an excess of HTFP. This latter rate should have been the same as for the first addition of enzyme, but the lower observed value could be due to the decomposition of the inactivator.

The value of K_I (1.4 mM) for HTFP is close to the values of 6.7 mM found for the protein fluorescence quenching for

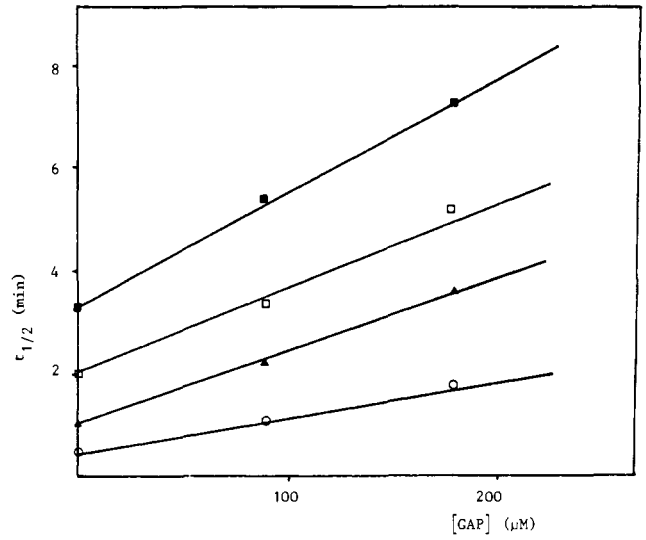


FIGURE 4: Protection against inactivation by GAP. Assays were carried out in the presence of HTFP at 0.21 (■), 0.26 (□), 0.84 (▲), and 1 mM (○). The concentrations of GAP were 0, 90, and 180 μM. Other experimental conditions were as described under Materials and Methods.

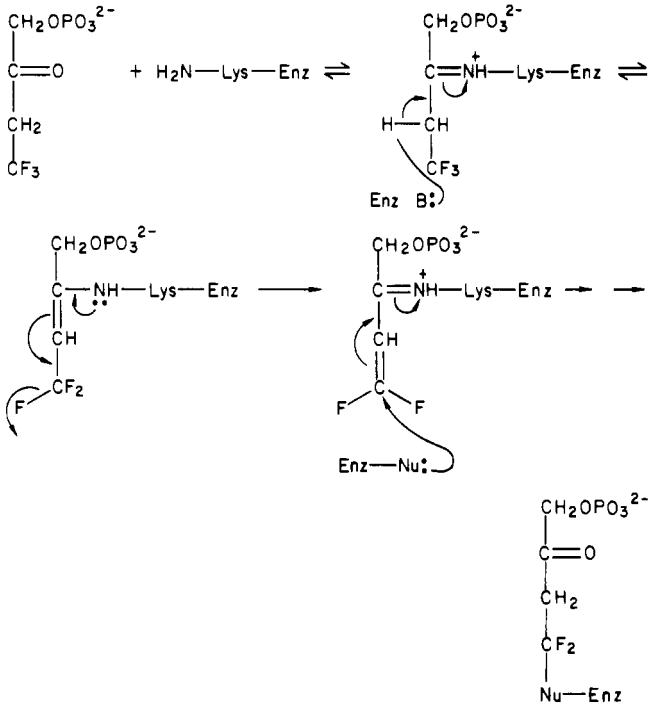
acetol phosphate (Rose & O'Connell, 1969) and of 0.1 mM found for the inactivation of aldolase with 2-keto-3-butenyl phosphate (K_I = 0.099 mM) (Motiu-De Grood et al., 1979). It is not clear whether k_{cat} (2.3×10^{-2} s^{-1}) determined with HTFP corresponds to the inactivation step or to the enzymatic transformation of the suicide substrate. The value of $k = 1.12 \times 10^{-1}$ s^{-1} found for the affinity labeling of aldolase by 2-keto-3-butenyl phosphate (Motiu-De Grood, 1979) could correspond to the inactivation step. Because of the electronegatively of the fluorine atoms vs. hydrogen, the inactivation rate should be higher in our case, so we may conclude that our value of 2.3×10^{-2} s^{-1} probably corresponds to the formation of the activated form of the suicide substrate.

The presence of both DHAP and GAP slowed down the inactivation rate. The protection by DHAP was expected since HTFP and DHAP compete for the same site on the enzyme. With GAP, the protective effect is consistent with the formation of a condensation product: 3-(trifluoromethyl)-3-deoxyfructose 1,6-bisphosphate. The characterization of this product needs further studies. The GAP effect on the aldolase inactivation contrasts with the result observed in bromopyruvate inactivation of 2-keto-3-deoxy-6-phosphogluconic aldolase, where pyruvic acid and 2-keto-3-deoxy-6-phosphogluconate protected but GAP had little or no effect on the inactivation (Meloche, 1967). The aldolase is known to catalyze the condensation of DHAP with pentose phosphate (Paoletti et al., 1980) and with glycolaldehyde and its phosphate (Byrne & Lardy, 1954) and the condensation of acetol phosphate with GAP (Rose & O'Connell, 1969). So, the condensation of HTFP with GAP by aldolase is not unprecedented.

A value of one to two F^- liberated by inactivated enzyme subunit has been calculated. This shows that HTFP is a very efficient suicide substrate. A mechanism that accounts for the results presented above is shown in Scheme III.

Aldolase contains eight cysteines per subunit (Lai, 1975): at positions 72, 134, 149, 177, 199, 237, 287, and 336. Cys-72 and -336 form a disulfide bridge on oxidation by oxygen in presence of o-phenanthroline–Cu^{II} (Lai, 1975). With DTNB, a total number of about seven cysteines was titrated in denaturing conditions. In the native enzyme about one very reactive and two slow reacting cysteines were determined

Scheme III



(Eagles et al., 1969). Later, the reactive cysteine was identified as Cys-237, and the modification of this residue did not inactivate the enzyme (Wong & Harper, 1982). The modification of cysteine of aldolase with disulfide monosulfoxides revealed four cysteines. Two were protected by a competitive inhibitor: hexitol 1,6-bisphosphate. After modification, the enzyme was still active (Steinman & Richards, 1970). When aldolase was treated with 2,4-dinitrochlorobenzene, one cysteine reacted and could be converted to dehydroalanine without loss of activity (Cremona, 1965).

With haloacetate, three cysteines were reactive (Szajani et al., 1970; Anderson & Perham, 1970). One whose modification led to inactivation was protected by phosphate ions (Szajani et al., 1970). With HTFP, the very reactive thiol group toward DTNB has disappeared. Cys-237, the most reactive thiol toward DTNB, could have reacted in the inactivation with HTFP. However, the modification of Cys-237 does not seem to impair the enzymic activity. It could be that the modification of Cys-237 impairs the enzymic activity only with certain modifying agent. We may not exclude that one of the three haloacetate-reactive cysteine leading to inactivation was modified during the inactivation with HTFP and that the reactivity of Cys-237 toward DTNB is strongly reduced by the HTFP inactivation. The rather confusing situation on the thiol reactivity awaits further structural information.

The extension of the study of HTFP as a suicide substrate may be envisaged. Indeed, DHAP plays a central role in the metabolism, and other enzymes acting on DHAP could be inactivated by HTFP. However, preliminary trials show that triosephosphate isomerase is only slowly inactivated by HTFP.

Registry No. HTFP, 93383-53-0; DHAP, 57-04-5; GAP, 142-10-9; FDP aldolase, 9024-52-6; 1-diazo-2-keto-4,4,4-trifluorobutane, 93383-54-1; 3,3,3-trifluoropropionyl chloride, 41463-83-6; diazomethane, 334-88-3.

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