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Alkylation of glyceraldehyde-3-phosphate dehydrogenase with haloacetylphosphonates

An unusual pH-dependence

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Two new alkylating reagents, chloro- and bromo-acetylphosphonate, were found to be very effective thiol-blocking reagents. The pH-dependence of the reaction of BAP with 2,4-dinitrothiophenol (25 °C, I 0.5) shows a tailing bell-shaped curve (with a plateau at high pH) characteristic of two ionizing groups: the thiol group (pK_a 3.2) and the phosphonate group (pK_{a2} 4.6). The rate constant for the reaction of the monoanionic inhibitor with dinitrothiophenolate (k_2 = 7 $M^{-1} \cdot s^{-1}$) is 120 times larger than that of the dianionic species. The haloacetylphosphonates were found to be irreversible inhibitors of glyceraldehyde-3-phosphate dehydrogenase from a variety of sources. They react with the active-site thiol group (Cys-149) and are half-site reagents with yeast glyceraldehyde-3-phosphate dehydrogenase. Thus, when two of the identical four subunits are modified the enzyme is catalytically inactive. The effects of pH (7–10), ^2H_2O and NAD$^+$ on the reaction with the yeast enzyme were examined in detail. NAD$^+$ enhances the alkylation rates. The second-order rate constant does not show a simple sigmoidal dependence on pH but rather a tailing bell-shaped curve (pK_a 7.0 and 8.4) qualitatively similar to that obtained with dinitrothiophenol. There is no significant solvent isotope effect on the limiting rate constants and a normal isotope effect on the two pK_a values. The results are consistent with the more reactive enzyme species containing a thiolate and an acidic group that may either donate a proton to the dianionic haloacetylphosphonate or orient the inhibitor.

INTRODUCTION

The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GPD, EC 1.2.1.12) catalyses the NAD$^+$-dependent oxidative phosphorylation of D-glyceraldehyde 3-phosphate (G3P) to produce 1,3-bisphosphoglyceric acid. The enzyme is a tetramer composed of four identical polypeptide chains, each with an essential cysteine residue. The enzyme displays the interesting property of half-of-the-sites reactivity (Levitzki & Koshland, 1976) with a variety of thiol-blocking reagents (Stallcup & Koshland, 1973*a*,*b*; Byers & Koshland, 1975). Thus, when two subunits are modified with a reagent such as iodoacetate, the yeast enzyme loses all of its catalytic activity (Stallcup & Koshland, 1973*a*). The extension of this half-site reactivity to the reaction with substrates, however, remains controversial (Malhotra & Bernhard, 1968; Trentham, 1971; Stallcup & Koshland, 1973*c*; Harrigan & Trentham, 1974; Cardon & Boyer, 1982). Another intriguing property of the enzyme is the role of NAD$^+$ in various steps of the catalytic mechanism. The reaction is known to involve formation of a thiohemiacetal with the active-site Cys-149 residue (Kanchuger *et al.*, 1979), oxidation by NAD$^+$ to form a thioester (Segal & Boyer, 1953; Velick & Hayes, 1953) followed by acyl transfer to inorganic phosphate. NAD$^+$ is required for this transfer of the phosphoglyceroyl group between Cys-149 and phosphate (Trentham, 1971; Byers *et al.*, 1979). In the absence of NAD$^+$, hydrolysis of the acyl-enzyme is at least 50000 times faster than phosphorolysis (Byers & Koshland, 1975). Thus NAD$^+$ functions both stoichiometrically as an oxidizing agent and catalytically in the overall reaction. In order better to understand the catalytic role of NAD$^+$ in the phosphorolytic deacylation of the enzyme and the possible role of subunit–subunit interactions in the catalytic mechanism we investigated the reaction of the enzyme with an active-site-directed irreversible inhibitor designed to mimic structurally the nucleophilic attack of inorganic phosphate on the acyl-enzyme.

MATERIALS AND METHODS

Materials

Yeast (*Saccharomyces cerevisiae*) GPD was obtained from Sigma Chemical Co. and shown to be homogeneous by electrophoresis on 10% polyacrylamide slab gels carried out in the presence of SDS (Laemmli, 1970). GPD from lobster muscle was prepared by the method of Buehner *et al.* (1974). The muscle GPDs from pig, chicken and rabbit and the enzymes from human erythrocytes and *Bacillus stearothermophilus* were obtained from Sigma Chemical Co., as were the other available substrates, buffers and reagents. Immediately before use the enzyme (10–30 mg/ml) was incubated for 20–30 min at pH 8.5 (50 mM-Bicine buffer containing 1 mM-EDTA, 0.5 M-NaCl and 25 mM-DTT). The sample was then passed through a small Sephadex G-50 column equilibrated with the standard pH 8.5 Bicine/EDTA buffer without DTT. The yeast enzyme concentration was determined from the absorbance at 280 nm with the absorption coefficient (corrected for the M_r of 145000) 1.35×10^5 $M^1 \cdot cm^{-1}$ (Krebs, 1955). The A_{280}/A_{260} ratio was 2.0–2.1, indicating little or no bound nucleotide.

Abbreviations used: BAP, bromoacetylphosphonate; CAP, chloroacetylphosphonate; Caps, cyclohexylaminopropanesulphonate; Ches, 2-(*N*-cyclohexylamino)ethanesulphonate; DNTP, 2,4-dinitrothiophenol; DTT, dithiothreitol; G3P, D-glyceraldehyde 3-phosphate; GPD, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); DTNB, 5,5′-dithiobis-(2-nitrobenzoate); NTB, 2-nitro-5-thiobenzoate; NTCB, 2-nitro-5-thiocyanobenzoate; SKIE, solvent kinetic isotope effect.

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2,4-Dinitrothiophenol (DNTP) was prepared by the following method. 2,4-Dinitrofluorobenzene (6.6 g) was treated with sodium sulphide ($Na_2S,9H_2O$, 8.7 g) in 30 ml of methanol for 10–20 min under N_2 and then poured into 400 ml of water to obtain crude product. The product was recrystallized in cyclohexane/acetone (1:1, v/v), m.p. 127–128 °C [lit. m.p. 128–130 °C (Kharasch & Parker, 1959)]. The absorption coefficient of DNTP at 404 nm is 1.61×10^4 $M^{-1} \cdot cm^{-1}$ and $pK_a = 3.18$ (± 0.01) from potentiometric titration.

Bromoacetylphosphonate (BAP) was prepared as the monocyclohexylammonium salt as described in the Appendix (Sparkes & Dixon, 1991). Chloroacetylphosphonate (CAP) was prepared by the reaction of Cl^- ion with BAP. BAP cyclohexylammonium salt (0.3 g) and LiCl (6 g) were dissolved in 20 ml of acetic acid and stirred at room temperature for 12 h. The solution was filtered to obtain crude product. Dilithium chloroacetylphosphonate was afforded by washing the crude product in methanol for 20 min and identified by ^1H n.m.r. (200 MHz, ^2H_2O) δ 4.63 (d, 2H, J 2 Hz); for BAP δ 3.80 (d, 2H, J 2 Hz). The concentrations of BAP and CAP solutions were checked by allowing them to react with a slight excess of GSH and determining the remaining free thiol groups by titration with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The concentrations were found to be in excellent agreement (± 1 %) with those based on the dry weight of the salt.

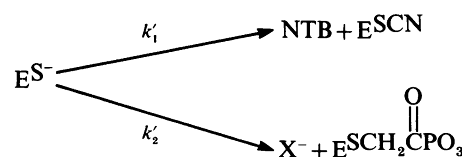
Kinetics

The buffer system usually consisted of 0.25 M-NaCl, 0.1 mM-EDTA and 25 mM buffer: sodium acetate (pH 3.5–5.6), Mes (pH 5.6–6.2), Hepes (pH 6.9–7.4), Bicine (pH 7.8–8.5), Ches (pH 9.1–9.8) or Caps (pH 10.2–11.4). Potentiometric titration and pH measurements were carried out on a Metrohm (Brinkmann) combitirator. For solutions in ^2H_2O, pD values were estimated from the formula: pD = pH (meter reading) + 0.41 (Covington *et al.*, 1968). The reactions of DNTP with alkylating reagents were monitored by the absorbance decrease at 404 nm. Rapid kinetics were monitored on a Dionex model D-110 stopped-flow spectrophotometer interfaced with a Biomation 810 transient digitizer and a strip-chart recorder. In general, kinetics were monitored on a Hewlett–Packard model 8452A diode array spectrophotometer or a Beckman 3600 spectrophotometer.

Competitive end-point technique

The reaction rates of the enzyme with alkylating reagents were determined by diluting samples from the reaction mixture into an assay solution containing either substrates (to determine activity loss) or 2-nitro-5-thiocyanobenzoic acid (NTCB) (to determine thiol group loss). For the very rapid reactions with BAP or CAP the kinetics were determined by measuring the end-point absorbance at 412 nm (due to 2-nitro-5-thiobenzoate) following the simultaneous addition of the inhibitor and NTCB to the enzyme (Scheme 1). k'_1 ($= k_1$[NTCB]) is the pseudo-first-order rate constant for the reaction with NTCB and k'_2 ($= k_2$[XAP]) is the pseudo-first-order rate constant for the reaction with BAP or CAP. These latter reagents compete with NTCB for the same site on the enzyme (Cys-149; see the Results section). The end-point absorbance is dependent on the relative pseudo-first-order rate constants (k'_1/k'_2) with the NTCB reaction serving as an 'internal clock'. The rate of this reaction was determined in a parallel experiment in the absence of XAP. The ratio of end-point absorbances (r) determined in the presence and absence of XAP is given by:

$$r = A_+/A_0 = [E^{SCN}]/[E]_0 = [NTB]/[E]_0 = k'_1/(k'_1+k'_2) \quad (1)$$



Scheme 1.

Re-arrangement yields the second-order rate constant for the alkylation reaction:

$$k_2 = (1-r)k_1[NTCB]/r[XAP] \quad (2)$$

As a test of this competitive end-point technique, rate constants were determined by this method for the reaction of CAP with GSH and were identical with those obtained by the conventional method of quenching portions of the reaction mixture into solutions containing NTCB or DTNB to determine remaining thiol groups.

RESULTS

Both BAP and CAP are effective irreversible inhibitors of GPD from a variety of species. Because of the high intrinsic reactivity of these compounds the reactions were carried out at low concentrations (0.5–20 μM-XAP). The loss of enzyme activity followed first-order kinetics ([XAP] $\gg$ [E]) for at least 4 half-lives with no evidence for saturation over this limited concentration range. The rate constants ($M^{-1} \cdot s^{-1}$, ± 4 %) for the reaction of BAP with GPD from various species (pH 7.5, 27 °C) are: 1.8×10^3 (apo-yeast), 2.3×10^4 (*B. stearothermophilus*), 2.4×10^4 (rabbit), 3.4×10^4 (chicken), 3.5×10^4 (human), 4.2×10^4 (pig), 5.5×10^4 (holo-yeast) and 8.5×10^4 (lobster). Although there is a 48-fold range in reactivity of BAP with enzymes from these different species the catalytic activities were similar. For example, the specificity constant ($k_{cat.}/K_m$) for the reaction of G3P with the enzyme from each of these species (1 mM-NAD^+, 25 mM-arsenate, pH 8.5) was found to be $3(\pm 1) \times 10^5$ $M^{-1} \cdot s^{-1}$. It is noteworthy that in the presence of saturating amounts of NAD^+ (5 mM) the yeast enzyme, at pH 7.5, is 30 times more reactive towards BAP (and 85 times more reactive at pH 8.3) than in the absence of NAD^+. Saturation with NADH (6.5 mM) resulted in only a modest (3-fold) rate enhancement. G3P inhibited the reaction with BAP. The amount of bound NAD^+ in the enzyme samples from the various species was estimated by measuring the NADH produced [$\epsilon_{340} = 6.3 \times 10^3$ $M^{-1} \cdot cm^{-1}$ (McComb *et al.*, 1976)] following the addition of 1 mM-G3P and 25 mM-arsenate. The fractional degrees of saturation (bound NAD^+/subunit) for the various GPDs were found to be < 0.01 for apo-yeast, 0.56 for human erythrocyte, 0.59 for *B. stearothermophilus*, 0.72 for lobster, 0.80 for rabbit, 0.88 for chicken and 0.90 for pig.

The reaction of BAP with the apo-GPD from yeast was carried out (at pH 7.5) at seven different temperatures (15–45 °C), yielding an apparent activation energy $E_a = 39.8(\pm 2.1)$ kJ/mol [$9.5(\pm 0.5)$ kcal/mol].

BAP is $2.16(\pm 0.04)$ times more reactive than CAP with both the apo- and holo-GPD from yeast. BAP and CAP were shown to react with the active-site thiol group of the yeast enzyme. When equivalent amounts of XAP (40 μM) and enzyme (10 μM) were incubated (for 5 min) there was a complete loss of enzyme activity. The undenatured enzyme showed no thiol groups (by titration with DTNB or NTCB) and the denatured enzyme (6 M-guanidinium chloride) showed one free thiol group per subunit (there are two cysteines per subunit). SDS/PAGE of the modified enzyme showed only monomers (M_r 35000), indicating no inter-subunit cross-linking.

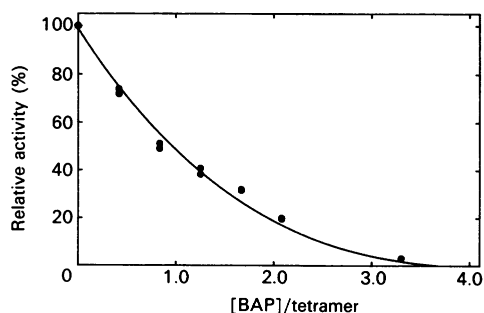


Fig. 1. Stoichiometry for inactivation of yeast GPD by BAP

The solid line is the theoretical curve assuming a binomial distribution of modified species with the di-, tri- and tetra-modified species being catalytically inactive and the mono-modified enzyme 41 % active.

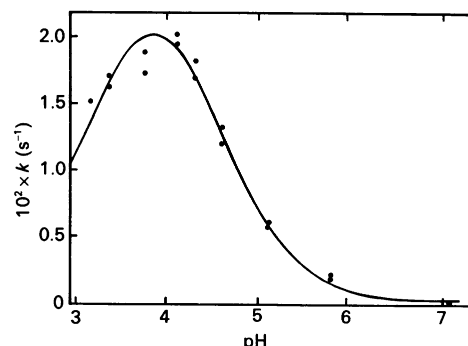
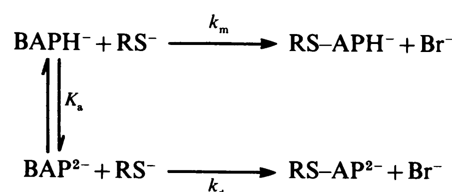


Fig. 2. Reaction of BAP with 2,4-dinitrothiophenol (27 °C, *I* 0.5)

Reactions were carried out under first-order conditions with BAP (4 mM) in excess. The data were fitted to eqn. (3) and yield pK_a(thiol) = 3.15($\pm$0.05), pK_{a2}(phosphonate) = 4.62($\pm$0.07), $k_m = 2.8(\pm0.1) \times 10^{-2}\ s^{-1}$ and $k_d = 2.4(\pm0.6) \times 10^{-4}\ s^{-1}$.



Scheme 2.

The stoichiometry for activity loss was determined by incubating various concentrations of BAP with a fixed excess amount of the enzyme (20 μM). The results, summarized in Fig. 1, indicate that BAP is a half-site reagent (see the Discussion section).

pH-dependence

Reaction of BAP with the model thiol, DNTP, was carried out in the range from pH 3.4 to 7.9 (Fig. 2). The kinetic pK_a of BAP derived from the pH profile, 4.6($\pm$0.1), is identical with the pK_a obtained from potentiometric titration. {This pK_a is sensitive to ionic strength. For example, when a solution of BAP was adjusted to pH 4.6 at an ionic strength of 0.5 and diluted with water to an ionic strength of 0.05 the pH increased by 0.45 unit. Extrapolation (of pK_a versus) to *I* approx. 0.02 yields a pK_a value of 5.2 [cf. the Appendix (Sparkes & Dixon, 1991)].} The second-order rate constant was obtained on the basis of Scheme 2 (assuming the thiolate is the only reactive species) and fitted to eqn. (3):

$$k_{obs.}/[BAP]_{tot.} = k_2 = k_m/(K_a/[H^+]+1) + k_d/([H^+]/K_a+1) \quad (3)$$

where k_m [= 7.0($\pm$0.3) $M^{-1}\cdot s^{-1}$] and k_d [= 0.06($\pm$0.02) $M^{-1}\cdot s^{-1}$] are second-order rate constants for the respective reactions of monoanionic and dianionic BAP with 2,4-dinitrothiophenolate. Thus the monoanionic species is more reactive by a factor of 120($\pm$30). With *p*-nitrothiophenolate the relative reactivities of the monoanion and dianion (k_m/k_d) is approx. 75. These rate constants, along with those of other thiol-blocking reagents, are summarized in Table 1. The reactivity of 2,4-dinitrothiophenolate with these reagents was found to be about 10 times less than that of *p*-nitrothiophenolate. The rate constant for the reaction of dianionic BAP with GSH (pK_a 9.0) is 760($\pm$17) $M^{-1}\cdot s^{-1}$. This is 1.6($\pm$0.1)-fold larger than the rate constant with CAP. An admittedly crude linear free-energy relationship for the reaction of dianionic BAP with the three thiols (2,4-dinitrothiophenolate, *p*-nitrothiophenolate and GSH) is

$$\log[k(M^{-1}\cdot s^{-1})] \approx 0.71pK_a - 3.5 \quad (4)$$

The pH-dependence for the reaction of NTCB with the holo-

Table 1. Rate constants for thiol alkylation

Reagent	Rate constant ($M^{-1}\cdot s^{-1}$)		
	DNTP*	Apo-GPD†	Holo-GPD†
Iodoacetate	0.097 ($\pm$0.002)	2.3 ($\pm$0.1)‡	64 ($\pm$5)‡
Iodoacetamide	0.325 ($\pm$0.003)	3.3 ($\pm$0.2) $\times 10^2$‡	12($\pm$1)‡
Bromopyruvate	5.5 ($\pm$0.3)	> 3.5 $\times 10^3$‡	–
CAP^{2-}§	4.5($\pm$0.7) $\times 10^{-2}$	3.3($\pm$0.2) $\times 10^2$	2.8($\pm$0.2) $\times 10^4$
BAP^{2-}‖	6($\pm$2) $\times 10^{-2}$	7.0($\pm$0.3) $\times 10^2$	5.4($\pm$0.2) $\times 10^4$
$CAPH^-$¶	2.5 ($\pm$0.1)	–	–
$BAPH^-$	7.0 ($\pm$0.3)	–	–
NTCB	–	2.5 ($\pm$0.1) $\times 10^3$**	9.9 ($\pm$0.2) $\times 10^3$

* Anion of 2,4-dinitrothiophenol.
† Yeast enzyme (pH 8.3, *I* 0.25, 27 °C).
‡ Monitored by activity loss.
§ Dianion of CAP.
‖ Dianion of BAP.
¶ Monoanion of CAP.
** This value is about 40 % of that obtained at an ionic strength of 0.03 (Byers & Koshland, 1975).

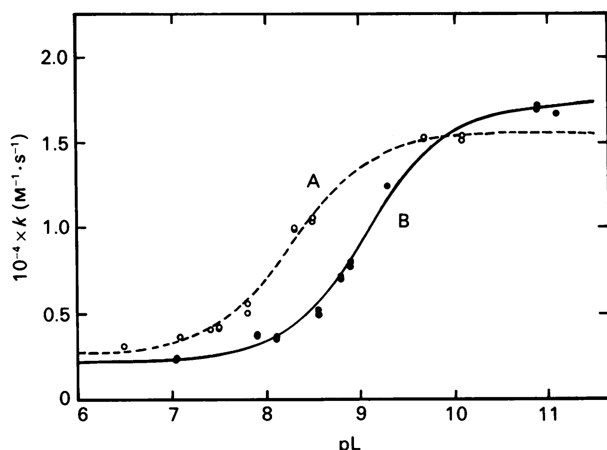


Fig. 3. Reaction of NTCB with holo-GPD from yeast in ^1H_2O (A) and ^2H_2O (B)

pK_a values are 8.28($\pm$0.04) (A) and 9.09 ($\pm$0.06) (B) and the limiting rate constants ($M^{-1}\cdot s^{-1}$) are 1.56 ($\pm$0.02)$\times 10^4$ and 2.6($\pm$0.2)$\times 10^3$ (A) and 1.73($\pm$0.03)$\times 10^4$ and 2.2($\pm$0.3)$\times 10^3$ (B).

enzyme is shown in Fig. 3. An interesting feature of this signmoidal curve [pK_a 8.28($\pm$0.04)] is that the second-order rate constant reaches a non-zero plateau at low pH. The solvent kinetic isotope effect (SKIE) on the low-pH plateau value is $^Dk = 1.2(\pm 0.2)$ and on the limiting rate constant at high pH is $^Dk = 0.92(\pm 0.04)$. A similar curve (not shown) is obtained with the apo-enzyme with pK_a = 8.85($\pm$0.05). The reaction of chloroacetamide and iodoacetamide with the yeast enzyme (results not shown) was carried out over a wide pH range (5.4–10.5) and yields a double sigmoidal curve similar to that obtained with the muscle enzyme (Polgár, 1975) and the *Escherichia coli* enzyme (Soukri *et al.*, 1989). In the case of the reaction of iodoacetamide with the yeast apo-enzyme the limiting rate at high pH was 15 times greater than the low-pH plateau rate and a pK_a of 8.8 (9.1 in ^2H_2O) was found. The SKIE on the limiting rate constant at high pH is $^Dk = 1.1(\pm 0.1)$.

The inactivation of yeast GPD in the presence of BAP was

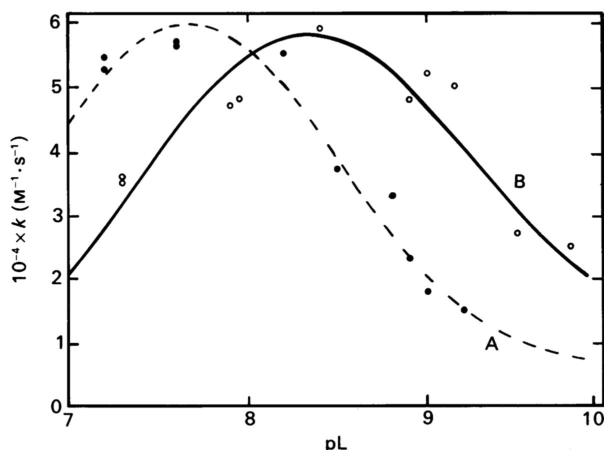


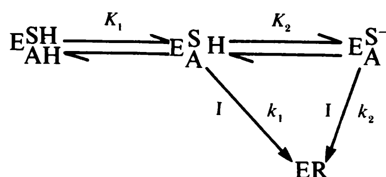
Fig. 4. Dependence of the second-order rate constant for the reaction of BAP with holo-GPD on pL

The theoretical curves are based on a fit to eqn. (5). In ^1H_2O (A) the parameters are pK_1 = 7.0($\pm$0.1), pK_2 = 8.35($\pm$0.05), $k_1 = 1.78(\pm 0.35)\times 10^6\ M^{-1}\cdot s^{-1}$ and $k_2 = 7(\pm 2)\times 10^3\ M^{-1}\cdot s^{-1}$. In ^2H_2O the corresponding parameters are 7.5($\pm$0.1), 9.05($\pm$0.1), 1.3($\pm$0.3)$\times 10^6\ M^{-1}\cdot s^{-1}$ and 1.1($\pm$0.3)$\times 10^4\ M^{-1}\cdot s^{-1}$.

followed over the pH range 7.2–9.6. The results with the holo-enzyme are illustrated in Fig. 4. Similar results were obtained with the apo-enzyme. In this pH range BAP exists exclusively (> 99%) as the dianion. The pH-dependence for the reaction is opposite that expected for the reaction with the thiolate form of the enzyme. The results suggest that there are at least two distinct enzyme species that react with BAP (similar curves are obtained with CAP). In addition to the ionization of the thiol [pK_a 8.35($\pm$0.05)], the ionization of an acidic group on the enzyme [pK_a 7.0($\pm$0.2)] also influences the reactivity. This group must be hydronated for optimal reactivity. This species is 250($\pm$90) times more reactive than the species in which this group has lost a hydron. There is no significant SKIE on the rate constant for the hydronated species nor on that of the ionized species present in the high-pL plateau region.

DISCUSSION

BAP and CAP are effective thiol-blocking reagents. It is noteworthy that they are somewhat less reactive than iodoacetamide towards model thiols such as 2,4-dinitrothiophenolate (Table 1). This may reflect the extensive hydration of the ketone moiety in XAP. [Using the Taft relationship for ketone hydration (Greenzaid *et al.*, 1967), the equilibrium constants for hydration of the dianionic species, BAP^{2-} and CAP^{2-}, are estimated to be 0.29 and 0.42 respectively. For the monoanionic species, $BAPH^-$ and $CAPH^-$, the hydration constants are estimated to be 148 and 207 respectively.] Both BAP and CAP are active-site-directed irreversible inhibitors of GPD from a variety of species. There is a greater species selectivity with the inhibitor than is apparent with the substrate (k_{G3P}). This species selectivity cannot totally be explained by the different amounts of NAD^+ bound to the various GPD samples. With the yeast enzyme, under pseudo-first-order conditions ([XAP] $\gg$ [E]), both thiol group loss and enzymic activity loss are complete and follow a single exponential decay. Thus, as in the case with DTNB (von Ellenrieder *et al.*, 1972), NTCB (Byers & Koshland, 1975), *p*-nitrophenyl acetate (Stallcup *et al.*, 1972) and glycidol phosphate (McCaul & Byers, 1976), XAP reacts independently with all four subunits of the enzyme. The stoichiometric relationship between activity loss and thiol group loss (Fig. 1), however, indicates that activity is completely lost when less than four thiol groups have been modified by BAP. For example, when an 'average' of two thiol groups are lost the enzyme has 25% of its original activity. However, since each thiol group reacts independently, the sample containing an average of two modified thiol groups is actually a mixture containing a binomial distribution of unmodified, mono-modified, di-modified, tri-modified and tetra-modified (E, ER, ER_2, ER_3 and ER_4) species. The best fit of the complete inactivation curve (Fig. 1) to a binomial distribution function is obtained when ER has 41% of the original activity and ER_2, ER_3, as well as ER_4, are completely inactive. Thus BAP is a half-of-the-sites reagent. When one of the subunits is modified by BAP a neighbouring subunit loses its catalytic activity as well. This is in contrast with the modification of the yeast enzyme with glycidol phosphate, which is a full-site reagent (McCaul & Byers, 1976). The enzyme modified with glycidol phosphate resembles the thiohemiacetal intermediate formed with G3P (Moody, 1984). G3P is known to react independently with all four subunits of the enzyme (Kirschner, 1971; Byers & Koshland, 1975). This is in contrast with the acylated enzyme, where only the doubly phosphoglyceroylated species is stable (Stallcup & Koshland, 1973*c*). Therefore, to the extent that the enzyme alkylated with XAP resembles the enzyme undergoing phosphorylytic deacylation, the half-site reactivity suggests that in the catalytic



Scheme 3.

turnover only half of the subunits may be functioning at a time.

In the reaction of BAP with the acidic model thiol, DNTP (pK_a 3.2), the pH-dependence shows two limiting rate constants characterized by the pK_a (4.6) of the electrophile. The monoanionic species is about 120 times more reactive than the dianionic species. A similar ratio was found with *p*-nitrothiophenolate. The greater reactivity of the monoanion is not surprising since the former species is more electrophilic than the latter [the Taft inductive substituent, σ^*, for $-PO_3^{2-}$ is 0.34 and for $-PO_3H^-$ is 1.93 (Shames & Byers, 1981*b*)]. A qualitatively similar profile is seen for the pH-dependence of the reaction of BAP with the enzyme (Fig. 4). Although this experiment was carried out over a pH range where BAP exists exclusively as the dianion, the observed rate constant still is dependent on two ionizing groups. Furthermore, the two pK_a values that characterize this pH-dependence are each larger than the pK_a of the inhibitor. The simplest scheme consistent with this is Scheme 3.

The general expression for the observed second-order rate constant for this scheme is:

$$k_{obs.} = \frac{k_1K_1[H^+]+k_2K_1K_2}{[H^+]^2+K_1[H^+]+K_1K_2} \quad (5)$$

where K_1 and K_2 are the macroscopic ('molecular') dissociation constants. In the reaction with NTCB, chloroacetamide or iodoacetamide the species $E^{S}_{A}H$ represents the tautomers which include an ion-pair. Persuasive evidence for this comes from the study by Soukri *et al.* (1989) with the *E. coli* enzyme. In the alkylation of the native enzyme with iodoacetamide they found a double sigmoidal dependence on pH (pK_a 5.5 and 8.6, with the high-pH limiting rate constant about 15-fold larger than the low-pH plateau rate constant). In the site-directed mutant enzyme, with asparagine replacing His-176, only a single sigmoidal curve (pK_a 9.3) was observed. The limiting rate constants at high pH were identical for the mutant and wild-type enzymes. The presence of a Cys–His ion-pair was originally suggested by Polgár (1975) in his alkylation studies with pig muscle GPD. [Ion-pairs have also been implicated in other thiol enzymes such as papain (Little & Brocklehurst, 1972; Polgár, 1977) and thymidylate synthase (Munroe *et al.*, 1978).]

A fit of the data (Fig. 4) to eqn. (5) yields $pK_2 = 8.4$, which is consistent with the pK_a of the essential thiol group in the holo-enzyme (observed in the titration with NTCB) and $pK_1 = 7.0$. The k_2 value for the holo-enzyme ($7\times10^3\ M^{-1}\cdot s^{-1}$) is 30-fold larger than the second-order rate constant for the reaction of BAP with a model thiol of pK_a 8.4, estimated from eqn. (4). This argues against the pK_1 reflecting a conformation change from a sterically inhibited form of the enzyme at high pH to one in which the cysteine is more accessible at low pH (see Bednar, 1990). Consistent with this is the observation, from deuterium exchange, that no detectable conformation change occurs in yeast GPD over the pH range 5.8–9.0 (Wrba *et al.*, 1990). The value of pK_1 is higher than that observed for the formation of the ion-pair. This probably reflects the involvement of a different residue. When this residue is protonated it may orient the inhibitor (via electrostatic interactions) or it may donate a proton to the inhibitor, enhancing its reactivity by a factor of about 250 ($k_1/k_2 = 1.7\times10^6\ M^{-1}\cdot s^{-1}/7\times10^3\ M^{-1}\cdot s^{-1}$).

Since, within experimental error, k_1 is identical in ^1H_2O and ^2H_2O [$^Dk = 1.3(\pm0.4)$], it is likely that hydron transfer in this step occurs before the rate-limiting nucleophilic attack. A similar situation may occur in the acylation of the enzyme with the physiological substrate, 1,3-bisphosphoglycerate. Protonation of the acyl phosphate residue will convert it from a relatively poor leaving group (with pK_a approx. 13) into a good leaving group (with pK_a 7). Conversely, in the phosphorylytic deacylation of the acyl-enzyme intermediate, proton removal from the attacking inorganic phosphate is expected to enhance its nucleophilicity by about a factor of 60 [$= 10^{\beta(\Delta pK_a)}$, where β ($= 0.3$) is the Brønsted coefficient for nucleophilic attack by phosphonates on thioesters (Shames & Byers, 1981*a*)].

Summary

The haloacetylphosphonates, BAP and CAP, are half-of-the-sites irreversible inhibitors of GPD. With model thiols the rate constant of the monoanionic haloacetylphosphonate is over two orders of magnitude greater than that of the dianionic species. A similar situation is seen with the enzyme. Although it is the thiolate species that reacts with the inhibitor, the reactivity is over two orders of magnitude greater when an enzymic 'group of pK_a 7' is protonated than when it is ionized. Both the apo- and holo-enzymes show similar behaviour, although the pK_a of the active-site Cys-149 is slightly lower in the holo-enzyme (8.4) than in the apo-enzyme (8.8). At pH 8.3 the holo-enzyme is about two orders of magnitude more reactive than the apo-enzyme. NAD^+ may facilitate the alkylation by electrostatic interactions or via an induced conformation change.

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APPENDIX

The preparation and properties of bromoacetylphosphonic acid

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Bromoacetylphosphonic acid, $Br–CH_2–CO–PO_3H_2$, was made by brominating dimethyl acetylphosphonate and de-esterifying with HBr. It proves to be a powerful alkylating agent, reacting rapidly with GSH, with a rate constant of about 6 $M^{-1}\cdot s^{-1}$ at pH 6.

INTRODUCTION

Acetylphosphonic acid, $CH_3–CO–PO_3H_2$, can have biochemical uses as an analogue of pyruvate or of phosphate. Harrison, Perham and Slater (cited by Ambrose & Perham, 1976) found it to be an effective inhibitor of pyruvate dehydrogenase, with a K_i of 4 μM, and Kluger & Pike (1977) reported that its monomethyl ester, which bears only a single charge at neutral pH, is an even better inhibitor, with a K_i of 0.05 μM. It therefore seemed of interest to make an alkylating derivative of this compound, so we developed a synthesis of bromoacetylphosphonic acid. This molecule, $Br–CH_2–CO–PO_3H_2$, possesses two electrophilic centres, the activated bromomethyl group, expected to react preferentially with polarizable nucleophiles, and a carbonyl group, expected to be sensitive to basic nucleophiles (cf. Edwards, 1956). They are closer to the phosphono group than in the previously made bromoacetyl phosphate, $Br–CH_2–CO–O–PO_3H_2$.

RESULTS

The bromoacetylphosphonic acid was synthesized by treating dimethyl acetylphosphonate, $CH_3–CO–PO(–O–Me)_2$, with Br_2, and completing the de-esterification with HBr in acetic acid. It proves to react rapidly with GSH, at about 6 $M^{-1}\cdot s^{-1}$ at pH 6.

EXPERIMENTAL

Synthesis

Dimethyl acetylphosphonate, $CH_3–CO–PO(–O–Me)_2$, was used as starting material, and was made by a modification of the method of Kabachnik & Rossiĭskaya (1945), as follows. Acetyl chloride (20 ml, 0.28 mol) was cooled to 0 °C. Trimethyl phosphite (30 ml, 0.25 mol) was added slowly with stirring and cooling, and the mixture was then heated to 80 °C for 5 min to drive off unchanged acetyl chloride. The crude product (39.2 g, 38 g theoretical) was used for bromination without purification. To a portion (38 g, 0.25 mol if pure) a few drops of Br_2 were added and the mixture was warmed until decolorization started (about 50 °C). The rest of the Br_2 (6.5 ml, 0.125 mol) was then added slowly with cooling to keep the temperature below 50 °C. Less than the theoretical quantity of Br_2 was used to avoid formation of dibromoacetylphosphonic acid. After this, 40 ml of 45 % HBr in acetic acid was added, and the mixture was kept at about 20 °C overnight [Cooke *et al.* (1953) had similarly de-esterified acetylphosphonic acid with anhydrous HBr]. The mixture was evaporated to dryness, and cooled with ice while it was dissolved in water and adjusted to pH 3.0 with cyclohexylamine. The solution was again evaporated to dryness, and traces of water were removed by re-evaporation from ethanol. The solid formed was washed by suspending and filtering off, five times with ethanol and five times with methanol, to remove cyclohexylamine salts of HBr, acetic acid and acetylphosphonic acid. A residue of 24.8 g remained (66 % based on the Br_2 added, 34 % on the trimethyl phosphite).

Characterization

On electrophoresis in 5 % acetic acid/0.5 % pyridine, pH 3.5, on Whatman 3MM paper cooled in white spirit, the product gave a single spot of 0.91 of the mobility of orthophosphate and 0.88 of the mobility of acetylphosphonic acid (with glucose as a standard of zero mobility) when stained for its carbonyl group by spraying with 5 mM-2,4-dinitrophenylhydrazine in 2 M-HCl, and for its ability to bind Fe^{3+} ions by spraying with $FeCl_3$ and sulphosalicylic acid solutions (the phosphate test of Wade & Morgan, 1953). [See Dixon & Sparkes (1974) for details of paper electrophoresis and staining.]

The crude product was stirred in methanol (50 ml per g) until dissolved; it crystallized on addition of diethyl ether. Elementary analysis gave C, 31.9; H, 5.7; N, 4.6 %; $C_8H_{17}BrNO_4P$ requires C, 31.8; H, 5.7; N, 4.6 %. Titration in water gives a pK of 5.2 and a molar mass of 299 g/mol (theoretical 302).

Preparation of the lutidine salt

Because of the difficulty of dissolving the cyclohexylamine salt, the salt with 2,6-lutidine was used for kinetic experiments. The cyclohexylamine salt was stirred with the sulphonic resin Zerolit 225 SRC13 (H^+ form) in water, and the suspension was filtered through a small bed of the same resin to obtain a solution of the free acid. This was adjusted to pH 3 with 2,6-lutidine, and was evaporated to dryness on a rotary evaporator. Ethanol was added and the solution again evaporated a few times until a