Short Communications

Thiol-Blocking Reagents and Phosphate Acetyltransferase Catalysis, and the Assessment of Protection by Adsorbed Molecules

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Phosphate acetyltransferase (EC 2.3.1.8) is shown to be unusually sensitive to thiol-blocking reagents, and one or more thiol groups probably catalyse the surface reaction. A novel method for assessing protection by adsorbed species is described. The adsorbed substrate molecules acetyl-CoA and phosphate are found to provide substantial protection against thiol-blocking reagents.

Reversible inhibition of the action of phosphate acetyltransferase (EC 2.3.1.8) by substrate analogues (Shimuzu *et al.*, 1970) and by products (Kyrtopoulos & Satchell, 1972*a,b*) has been carefully studied, but there has been little systematic examination of inhibition by group-specific inhibitors. We now report briefly on the effects of four reagents that attack thiol groups. We also present a new method for estimating protection against these and similar reagents. It has been reported previously that phosphate acetyltransferase derived from *Clostridium kluyveri* or from *Escherichia coli* B is sensitive to *p*-chloromercuribenzoate, but few details are available (Shimuzu *et al.*, 1969; Bergmeyer *et al.*, 1963). Our work refers to the *Cl. kluyveri* enzyme only.

Experimental

In this study to examine the effects on the enzyme of reagents that attack thiol groups, we chose the velocity of the reverse reaction (1b) of the phosphate acetyltransferase-catalysed equilibrium (1):

to monitor the enzyme's activity. This choice avoids the complication of one of the substrates containing a thiol group; tests showed that the use of the forward reaction to monitor the inhibition was complicated by the presence of the CoA thiol group, which led to unwanted protection effects. Of course, as reaction (1b) proceeds CoA accumulates, but the use of initial velocities minimizes this difficulty. Reaction (1b) was followed as before by u.v. spectroscopy at 235 nm (Kyrtopoulos & Satchell, 1972*a,b*). All experiments were at 25°C. Our procedure was to incubate a standard quantity of enzyme with various concentrations of inhibitor for known periods of time, and then to test its activity in catalysing reaction (1b) and to compare this activity with that found in the absence of inhibitor. In some experiments enzyme was incubated

with inhibitor plus either acetyl-CoA or phosphate ions to test the protective action of these substrates.

It is known (Kyrtopoulos & Satchell, 1973) that the enzyme rapidly and spontaneously loses activity when used in solution in the absence either of thiol reducing agents (e.g. dithiothreitol) or of certain salts (e.g. $(\text{NH}_4)_2\text{SO}_4$). In the present experiments inclusion of a reducing thiol was clearly undesirable, but sufficient $(\text{NH}_4)_2\text{SO}_4$ was always present in the incubation mixture to diminish spontaneous inactivation at 25°C to a value small compared with that produced by the added inhibitors. An appropriate correction for the spontaneous inactivation was, however, always made. The thiol-blocking reagents studied included representatives from most of the important types of this group of inhibitors (Webb, 1966). All the inhibitions were carried out in Tris buffers (pH 8.32) and at a constant ionic strength of 0.4 M in the final reaction mixture. In a typical experiment a solution containing enzyme, inhibitor, buffer and $(\text{NH}_4)_2\text{SO}_4$ (1 ml total) was incubated, and then the enzyme-catalysed reaction was initiated by the successive rapid additions of standard solutions of acetyl-CoA and sodium hydrogen phosphate in buffer (final volume 3 ml). When either substrate was included in the incubation mixture it was omitted in this initiation process. Separate experiments showed that absorbance changes arising from the reaction of the inhibitors with the CoA product were sufficiently small at the concentrations and reaction times employed to be negligible compared with the absorbance changes arising directly from reaction (1b). Observed initial velocities (v_0) were reproducible to within $\pm 3\%$.

Results and discussion

Typical results are shown in Table 1. All four inhibitors, if left in contact with the enzyme for sufficiently long, lead to its complete (>99%) inactivation. However, their relative efficiencies, in terms of speed of action at a given concentration, differ.

Table 1. *Inhibition of phosphate acetyltransferase activity by thiol-blocking reagents*

The conditions used were as follows: $[E]_0$, 8.25 nM (in the incubation mixture); $[NH_4^+]$, 18 mM (in the incubation mixture); Tris buffer, pH 8.32, 0.2 M; temperature, 25°C; $[acetyl-CoA]_0$, 0.093 mM; $[phosphate]_0$, 0.101 M; $[Na^+]$, 0.202 M; ionic strength, 0.4 M. v_0 is the initial velocity ($\text{mol} \cdot \text{l}^{-1} \cdot \text{min}^{-1}$). Fractional activity is calculated from v_0 values after appropriate correction for the small amount of spontaneous inactivation (for details see Kyrtopoulos, 1972).

[Inhibitor] (mM)	Incubation period (min)	$10^6 v_0$		Fractional activity	
		Iodoacetate	Iodoacetamide	Iodoacetate	Iodoacetamide
0.28	0	13.4	13.4	1.00	1.00
0.28	1.5	13.2	12.0	0.98	0.90
0.28	30	7.9	8.7	0.73	0.79
0.28	30*	9.8	9.6	0.90	0.88
0.28	30†	8.9	9.7	0.82	0.89
0.28	60	5.2	5.7	0.52	0.56
0.096	30	9.4	9.5	0.88	0.88
0.58	30	5.0	4.9	0.46	0.46
		Iodosobenzoate	<i>p</i> -Chloro- mercuribenzoate	Iodosobenzoate	<i>p</i> -Chloro- mercuribenzoate
0.00058	0	13.4	13.4	1.00	1.00
0.00058	1.2	—	11.1	—	0.84
0.00058	5.0	9.7	5.5	0.77	0.45
0.00058	10	8.3	3.1	0.66	0.24
0.00058	30	7.0	2.5	0.64	0.23
0.00058	30*	9.0	—	0.83	—
0.00058	30†	8.0	8.9	0.73	0.81
0.00058	30‡	—	2.7	—	0.25
0.00058	60	6.1	1.9	0.60	0.18
0.014	10	4.5	—	0.37	—
0.014	30	1.9	—	0.17	—
0.028	10	2.2	0.0	0.18	0.00
0.280	10	0.5	—	0.04	—
0.280	1.2	0.0	—	0.00	—

* Incubation with 0.303 M-sodium hydrogen phosphate.

† Incubation with 0.278 mM-acetyl-CoA.

‡ Incubation with inhibitor followed by incubation with 0.139 mM-acetyl-CoA for 2 min.

Fig. 1. *Inactivation of phosphate acetyltransferase by thiol-blocking reagents*

General conditions were as given in Table 1. The inhibitors used were: ○, 0.28 mM-iodoacetamide; ●, 0.28 mM-iodoacetate; □, 580 nm-*o*-iodosobenzoate; ■, 580 nm-*p*-chloro-mercuribenzoate.

(i) *Iodoacetate and iodoacetamide*. These substances, which operate by alkylating thiol (and other) groups, possess very similar efficiencies as inhibitors of phosphate acetyltransferase (Fig. 1). Incubation in the presence of phosphate ions or of acetyl-CoA (substrate species adsorbed by the enzyme) leads to protection (Table 1). We have used the following procedure to obtain a quantitative measure of the protection afforded by the presence on the enzyme of an adsorbed species. Assuming second-order kinetics (see, however, Kitz & Wilson, 1962), the initial-rate equation for the inactivation process in the absence of protecting substance can be written as eqn. (2):

$$R_0 = k_0[E]_0[I] \quad (2)$$

where k_0 is a rate constant and I represents inhibitor. With a protective substance adsorbed on the enzyme, eqn. (2) becomes eqn. (3):

$$R = k_0[E][I] + k([E]_0 - [E])[I] \quad (3)$$

where $[E]$ is the free enzyme concentration and k is the rate constant for the reaction of inhibitor with the enzyme carrying the protecting substance. If complete protection is afforded, $k = 0$; if none, $k = k_0$. The amount of protection, as reflected in the ratio k/k_0 , is given by eqn. (4):

$$k/k_0 = \left(\frac{R}{R_0} - \frac{[E]}{[E]_0} \right) / \left(1 - \frac{[E]}{[E]_0} \right) \quad (4)$$

[For another approach see Anderton & Rabin (1970).] The ratio R/R_0 will be approximately equal to the ratio of the amounts of inactivation produced, after a given brief period of incubation, in the presence and in the absence of inhibitor respectively. $[E]/[E]_0$ can be calculated from the concentration of protective substance and its appropriate Michaelis (dissociation) constant (Kyrtopoulos & Satchell, 1973). In this way we calculate that either an adsorbed phosphate ion or an adsorbed acetyl-CoA species provides effectively complete (i.e. $k/k_0 = 0.0 \pm 0.07$) protection against the present inhibitors. This result suggests that the inhibited group(s) involved is very close to the binding sites of both substrates and close therefore to the region in which the acetyl group is actually transferred.

(ii) *o*-Iodosobenzoate. Our results show that phosphate acetyltransferase is more strongly inhibited by iodosobenzoate than are most enzymes (Webb, 1966); there is a substantial rapid loss of activity followed by a slower fall. The involvement of at least one thiol group is therefore suggested. Again the protection results indicate that the presence of adsorbed phosphate ($k/k_0 = 0.2 \pm 0.1$) or acetyl-CoA ($k/k_0 = 0.0 \pm 0.03$) leads to substantial protection against the initial deactivation, so that the group involved is likely to be adjacent to the seat of the enzyme-catalysed reaction. (The conclusions from the protection experiments will be less firm for this inhibitor, since a larger fraction of the inhibition reaction had taken place before v_0 was determined.)

(iii) *p*-Chloromercuribenzoate. This very specific thiol-blocking reagent exists in solution largely as the hydroxy form, which mercurates thiols. Phosphate acetyltransferase is very strongly affected (Fig. 1), and more so than most enzymes. As with *o*-iodosobenzoate, there are two phases of inhibition, one rapid, the other slower. It seems likely that the rapid inhibition arises from attack on the thiol

group(s) already identified as lying close to the seat of the enzyme-catalysed reaction. It is noteworthy that the effect of *p*-chloromercuribenzoate is readily reversed by the presence of CoA. As the concentration of this compound builds up during an inhibited run it affords increasing protection, presumably owing to the inhibitor's preferential reaction with the CoA thiol group. Our results, compared with those of Shimizu *et al.* (1969), show that the *Cl. kluyveri* enzyme is far more susceptible to *p*-chloromercuribenzoate than is the *E. coli* B enzyme.

In all we conclude therefore that one or more thiol groups may be essential to the catalytic function of phosphate acetyltransferase. (The inhibited groups are certainly very susceptible to thiol-group inhibitors.) Since both substrates protect the enzyme efficiently, and since no groups with pK_a values typical of thiols were detected in our earlier pH analysis of substrate binding (Hibbert *et al.*, 1971), it is suggested that the important role of the thiol group(s) lies in its catalysis of the reaction between the adsorbed substrates. A group with pK_a approx. 8.7–9.0 was indeed shown earlier (Hibbert *et al.*, 1971) to affect the rate of this reaction. Our studies thus complement each other.

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