Arylthallium(III) reagents for protein modification

Inhibition of lactate dehydrogenase from various sources by *o*-carboxyphenylthallium(III) bistrifluoroacetate

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The use of organothallium compounds for protein/macromolecule modification and as probes for n.m.r. and fluorescence is introduced. Lactate dehydrogenase from a number of species was rapidly and specifically inhibited by *o*-carboxyphenylthallium(III) bistrifluoroacetate and *p*-methylphenylthallium(III) bistrifluoroacetate. Inhibition of rabbit muscle lactate dehydrogenase by *o*-carboxyphenylthallium(III) bistrifluoroacetate was time-dependent and not reversible by gel filtration. A small degree of re-activation was possible by incubation with dithiothreitol. The time course of the inactivation kinetics showed two phases, only the first, and faster, of which was efficiently prevented by the presence of cofactor, NADH. Inhibition rates depended on the structure of the thallium reagent, its concentration and the temperature. No significant inhibition was found by thallos acetate or thallic trifluoroacetate. Saturation kinetics were observed for the inhibition by *o*-carboxyphenylthallium(III) bistrifluoroacetate of the pig heart enzyme. The possibilities of various cross-linking activities of these reagents are addressed. Mechanisms of the inhibition are discussed.

The n.m.r. properties of the stable isotope ^{205}Tl (spin $\frac{1}{2}$, 70.48% natural abundance) have suggested the use of the Tl^+ ion to probe its environment when bound to biological molecules, e.g. pyruvate kinase (Kayne & Reuben, 1970; Reuben & Kayne, 1971), $\text{Na}^+ + \text{K}^+$ -activated ATPase (Grisham *et al.*, 1974), the ionophore lasalocid, X-537A (Briggs *et al.*, 1980), and gramicidin (Turner *et al.*, 1982; Hinton *et al.*, 1982). Such studies are possible because Tl^+ can often replace K^+ ions (Britten & Blank, 1968; Inturisi, 1969*a,b*; Robinson, 1970; Kayne, 1971; Bostian *et al.*, 1975, 1982). In addition, Tl^+ has been suggested as a fluorescence probe (Cornelius *et al.*, 1974; Mannors *et al.*, 1971).

To our knowledge, there has been no use made of arylthallium(III) reagents in biological chemistry, although recently the thallic oxidation state has been used to provide insight into transferrin by the use of ^{205}Tl n.m.r. spectroscopy (Bertini *et al.*, 1983). Consequently, we have considered aryl-

thallium(III) derivatives (CPT and MPT) as potential modifiers of biological molecules. Arylthallium(III) derivatives are readily prepared (McKillop *et al.*, 1969) and have been of great value as intermediates in synthetic organic chemistry.

We reasoned that Tl(III) would be strongly electrophilic and should, by analogy with the widely used organomercurials, show a marked preference for 'softer' sites (e.g. cysteine and histidine residues). Derivatives CPT and MPT have two easily replaceable ligands (trifluoroacetoxy groups)

Abbreviations used: CPT, *o*-carboxyphenylthallium(III) bistrifluoroacetate; MPT, *p*-methylphenylthallium(III) bistrifluoroacetate.

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and are in principle reagents capable of cross-linking either inter- or intra-molecularly.

The present study reports the inhibition of lactate dehydrogenase (EC 1.1.1.27) from a number of species by derivatives CPT and MPT.

Materials and methods

Materials

L-Lactate dehydrogenases from rabbit skeletal muscle (type XI; specific activity 830 units/mg), from pig heart (type XVIII; specific activity 525 units/mg), from lobster tail (type XXIII; specific activity 490 units/mg) and from chicken heart (type VIII; specific activity 538 units/mg) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), as were NADH, NAD⁺ and sodium pyruvate. Potassium phosphate and thallous acetate were from BDH Chemicals (Poole, Dorset, U.K.). Thallium trifluoroacetate, CPT and MPT were gifts from Mr. F. M. Said and had been synthesized by the method of McKillop *et al.* (1971). Sephadex gel-filtration media were from Pharmacia Fine Chemicals (Uppsala, Sweden).

Assay of lactate dehydrogenase activity

To initiate the assay reaction, enzyme (50 μ l) was added to a mixture containing NADH (0.122 mM) and sodium pyruvate (0.666 mM) in 27.5 mM-phosphate buffer, pH 7.4, at 25.0°C, to give a total volume of 3.0 ml. The reaction was followed spectrophotometrically at 340 nm. To prepare assay media concentrated stock solutions of NADH and sodium pyruvate were prepared freshly before use in phosphate buffer, pH 8.0, and phosphate buffer, pH 7.4, respectively. One unit of lactate dehydrogenase will reduce 1 μ mol of pyruvate to L-lactate in 1 min at pH 7.4 at 25°C.

Modification conditions

Typical conditions for thallium modifications of lactate dehydrogenase were as follows. CPT and lactate dehydrogenase (from an approx. 1 μ M stock solution in phosphate buffer) were incubated together at 25°C in a total volume of 2.0 ml made up with 30 mM-phosphate buffer, pH 7.4. The CPT was added as a small portion ($\leq 15 \mu$ l) from a concentrated stock solution in dimethyl sulphoxide to initiate reactions. The final concentration of this co-solvent in the incubation mixture was kept at $\leq 1.0\%$ (v/v), at which concentration it had no discernible effect on the assay velocity of lactate dehydrogenase. Samples (50 μ l) were removed and assayed for the enzyme activity at appropriate intervals. Appropriate controls were studied under identical conditions but in the absence of thallium reagents etc.

Kinetic procedures

Spectrophotometric studies were performed with a Pye-Unicam SP.8100 u.v.-visible spectrophotometer with its cuvette compartment thermostatically maintained at 25°C by means of circulating water from a Churchill thermoregulator. Buffer solutions were prepared from AnalaR-quality materials with glass-distilled deionized water and the use of a Philips PW 9409 digital pH-meter. Data analyses were performed by means of a least-squares linear-regression programme on a Exidy Sorcerer minicomputer.

Solubility properties of thallium reagents

Thallous acetate is highly soluble in phosphate buffer, pH 7.4, with no sign of precipitation at 1 mM, but about 0.125 mM-thallic trifluoroacetate was precipitated in phosphate buffer, pH 7.4, at 25°C. CPT was soluble up to 0.75 mM under these conditions, whereas MPT was less soluble and we found it advisable to work at no more than 0.025 mM with this reagent.

Results

CPT was found to induce a time-dependent inhibition of rabbit muscle lactate dehydrogenase (at pH 7.40 at 25°C). A typical activity-time profile is shown in Fig. 1(a). There are two phases of inhibition, and these are more clearly seen by plotting the data semi-logarithmically, as shown in Fig. 1(b). In the latter stages of the inhibition reaction, in which the enzyme activity is ultimately decreased to zero, within experimental error, apparent first-order kinetics are followed. For this section of the time-dependence, the reaction can be described by an apparent first-order rate constant k_{obs} . [$= -2.303 \times$ final slope of plot of $\log(\text{activity remaining})$ versus time]. However, this portion of the semi-logarithmic plot does not extrapolate back through the control velocity (see Fig. 1b). The control velocity and the point at 0.5 min lie above the extrapolated line. The final-phase reaction actually extrapolates back to approx. 75% of the velocity of the control reaction (i.e. that in the absence of CPT). Apparently, there is a rapid initial reaction of CPT with lactate dehydrogenase leading to loss of approx. 25% of activity within the first 2–3 min, and this is followed by a much slower loss of the remaining activity over the next 20 min or so. Some parameters governing the slower reaction (described by k_{obs} values) are reported below.

Under the above conditions, with $[\text{CPT}] \gg [\text{E}_0]$, where $[\text{E}_0]$ is the initial concentration of enzyme, values of k_{obs} were found to be independent of $[\text{E}_0]$, within the limits of the experiment. For a CPT concentration of 0.5 mM, the values of k_{obs} were $0.166 \pm 0.007 \text{ min}^{-1}$, $0.182 \pm 0.012 \text{ min}^{-1}$ and

Fig. 1. (a) Plot of observed assay velocity for samples removed from a mixture of rabbit muscle lactate dehydrogenase and CPT in 30 mM-phosphate buffer, pH 7.4, at 25°C, and (b) plot of log (activity remaining) versus time for the data used to construct (a) (a) Plot of observed assay velocity (○) for samples removed from a mixture of rabbit muscle lactate dehydrogenase and CPT (0.5 mM) in 30 mM-phosphate buffer, pH 7.4, at 25°C. Points are experimental; the continuous line is a smooth curve to assist visualization. Also shown are the data for the control system (□), which was identical in components but for the absence of CPT and lost no detectable activity during the course of the experiment. The concentration of dimethyl sulphoxide in both cases was 0.75% (v/v). (b) Plot of log (activity remaining) versus time for the data used to construct (a). The continuous line after 2 min was obtained by least-squares linear-regression analysis of the data (omitting the points at zero time and 0.5 min). The points are experimental, and that quoted as zero time refers to the observed mean velocity of the control, which was stable to $\pm 0.83\%$ over the time period (see a).

$0.191 \pm 0.012 \text{ min}^{-1}$ at enzyme concentrations of $0.274 \mu\text{M}$, $0.137 \mu\text{M}$ and $0.0686 \mu\text{M}$ respectively.

Values of k_{obs} as a function of [CPT] are plotted in Fig. 2, which shows that the reaction is first-order in [CPT]. Thus the rate law for the inhibition observed after approx. 2 min incubation with CPT under the above conditions (pH 7.4, 25°C, [CPT] in the range 0.025–0.625 mM) is given by eqn. (1):

$$\text{Velocity} = k_{\text{Tl}}[\text{E}_0][\text{CPT}] \quad (1)$$

The value of k_{Tl} was determined to be $413 \pm 13 \text{ M}^{-1} \cdot \text{min}^{-1}$ by least-squares regression analysis of the data in Table 1, by assuming the rate law (1) above.

The k_{obs} rate constant for inhibition (measured at $t > 2 \text{ min}$) by CPT showed a marked temperature-dependence, with values of k_{obs} (above conditions, other than temperature) as follows (temperatures in parentheses): $0.036 \pm 0.002 \text{ min}^{-1}$ (4°C), $0.180 \pm 0.011 \text{ min}^{-1}$ (25.0°C) and $0.850 \pm 0.039 \text{ min}^{-1}$ (37.0°C) for [CPT] = 0.5 mM.

Effects of other organothallium compounds on lactate dehydrogenase

Thallous acetate showed no effect on enzyme activity when incubated at concentrations up to 1 mM (at pH 7.4 at 25°C) with rabbit muscle lactate dehydrogenase for periods up to 42 min.

Thallic trifluoroacetate showed no significant inhibition over 20 min at 0.075 and 0.125 mM (at pH 7.40 at 25°C). It may have given some inhibi-

Fig. 2. Dependence on concentration of CPT of the pseudo-first-order rate constant for inhibition (k_{obs}) of rabbit muscle lactate dehydrogenase in 30 mM-phosphate buffer, pH 7.4, at 25°C

Points are experimental and refer to data obtained for different batches of enzyme and on a number of different days. The continuous line was obtained by least-squares linear-regression analysis of the data and assuming that the line should pass through the origin. The slope (k_{Tl}) was $413 \pm 13 \text{ M}^{-1} \cdot \text{min}^{-1}$. Sets of points for a particular day and enzyme batch are grouped under a common symbol for each set. Two extra points at 0.5 mM-CPT have been omitted for clarity of presentation.

tion at 0.25 mM (approx. 25% inhibition after 19 min), but at this concentration there were noticeable precipitation problems for this compound.

Inhibition was observed with the derivative

Table 1. Rates of inhibition of pig heart lactate dehydrogenase by CPT in 30 mM-phosphate buffer, pH 7.4, at 25°C

Except where noted the enzyme concentration in the incubation mixture was 1.145 µg/ml.

Concn. of CPT (mM)	$k_{\text{obs.}}$ (min ⁻¹)
1.0*	0.368 ± 0.011
1.0	0.379 ± 0.009
0.75	0.320 ± 0.010
0.50	0.237 ± 0.005

* The enzyme concentration was 0.594 µg/ml.

MPT. At equal concentrations (0.025 mM) of thallium(III) species, chosen to avoid any precipitation problems, and equal enzyme concentrations etc. the results shown in Fig. 3 were obtained. Under these identical conditions of relatively low concentrations of thallium(III) reagent, MPT had a value of $k_{\text{obs.}} = 0.122 \pm 0.006 \text{ min}^{-1}$, which is 3.5-fold greater than that ($0.0345 \pm 0.0028 \text{ min}^{-1}$) for CPT. The $k_{\text{obs.}}$ value again refers to the latter stages of reaction, as the point at 0.5 min was excluded from the correlation (see below) for each derivative.

Effects of cofactors

The effect of the presence of the NADH cofactor of rabbit muscle lactate dehydrogenase on the reaction with CPT (at pH 7.40 at 25°C) was studied, and results are shown in Fig. 4(a). Clearly, the rate of inhibition is significantly altered by the presence of 1.0 mM-NADH at this concentration of CPT. The protective effect of NADH is complex, however. To obtain rate constants, $k_{\text{obs.}}$, for the latter phase of the time-dependence in the presence of NADH, account was taken of the slow linear (approx. 5.6% per 10 min) loss in activity with time of the control enzyme system (i.e. enzyme plus NADH and no CPT reagent).

In Fig. 4(b), the data of Fig. 4(a) are replotted as log(activity remaining, normalized and corrected) versus time with correction for the NADH-induced inactivation of lactate dehydrogenase, and the data are normalized to allow direct comparison with data from Fig. 1, also shown normalized on this plot. Both sets of data were obtained with the same enzyme sample and on the same afternoon, and the difference in control assay velocities (extrapolated to zero time for lactate dehydrogenase in the presence of 1 mM-NADH and the mean of the velocities over 20 min in its absence) was 1.4%. Whereas in the absence of NADH (lower curve) the fast phase leading to loss of approx. 25% of activity is clearly evident, the presence of 1 mM-NADH introduces a lag in the loss of the enzyme activity caused by CPT under these conditions.

Fig. 3. Semi-logarithmic plots for the inhibition by CPT (□) and MPT (○) of rabbit muscle lactate dehydrogenase in 30 mM-phosphate buffer, pH 7.4, at 25°C

Points are experimental; the continuous lines are theoretical, obtained by using the first-order rate equation and $k_{\text{obs.}}$ values given in the text.

The lag corresponds in duration to the length of time taken for the corresponding CPT reaction to enter the apparent first-order region (i.e. approx. 2–3 min) in the absence of NADH. The latter, slower, phase of the kinetics appears to be little affected by the presence of 1 mM-NADH, as the final slopes are almost identical. If anything, $k_{\text{obs.}}$ in the presence of NADH ($= 0.200 \pm 0.003 \text{ min}^{-1}$) may even be slightly greater than in its absence ($k_{\text{obs.}} = 0.188 \pm 0.003 \text{ min}^{-1}$). It is difficult to assess accurately the degree of protection afforded by NADH in the early stages of the CPT inhibition, but comparison of initial tangents to the semi-logarithmic curves of Fig. 4(b) (see broken lines) shows that in the first 2 min the expected percentage inhibition in the absence of NADH would be 70%, whereas in the presence of 1 mM-NADH the corresponding value is 21%.

The presence of 1 mM-NAD⁺ had no discernible effect on the rate of inhibition (at 25°C at pH 7.4) of rabbit muscle lactate dehydrogenase by 0.75 mM-CPT, the inhibition being >99% complete in 10 min and both progress curves appearing to be identical.

Results with lactate dehydrogenases from different animal sources

The inhibition by CPT of lactate dehydrogenases from a number of sources was screened. Reaction with pig heart lactate dehydrogenase was rapid at pH 7.4 (at 25°C) and was complete within approx. 17 min for CPT concentrations from 0.5 to 1.0 mM. The higher CPT concentration was possible because the inhibition was complete (as determined by assay velocity) long before precipitation problems with the system were encountered (usual-

Fig. 4. (a) Plot of the observed assay velocity versus time for the reaction of CPT with rabbit muscle lactate dehydrogenase in the presence of NADH in 30 mM-phosphate buffer, pH 7.4, at 25°C, and (b) plot of log(normalized remaining activity) versus time for the inhibition by CPT of rabbit muscle dehydrogenase in the presence and in the absence of NADH

(a) Plot of the observed assay velocity (○) versus time for the reaction of CPT (0.5 mM) with rabbit muscle lactate dehydrogenase in the presence of NADH (1 mM) in 30 mM-phosphate buffer, pH 7.4, at 25°C. The data for the control system (enzyme plus 1.0 mM-NADH) are also shown (□). The broken line (---) represents the data for the corresponding reaction in the absence of NADH (taken from Fig. 1a) for comparison. (b) Plot of log (normalized remaining activity) versus time for the inhibition by CPT of rabbit muscle lactate dehydrogenase in the presence (○) and in the absence (□) of 1.0 mM-NADH. Points are experimental; continuous lines at times longer than 4 min were derived by least-squares linear-regression analysis of the data, omitting the initial time values for each case.

ly at approx. 60 min). Overall the inhibition time course was also biphasic but with an initial lag before the final stage: the latter again gave a linear plot of log(activity remaining) versus time with rate constant, k_{obs} , (see Fig. 5). The initial phase is seen as a deviation but in the opposite sense to that observed for the rabbit muscle enzyme. The dependences of k_{obs} on the concentrations of enzyme and of CPT are summarized in Table 1. The value of k_{obs} was independent of the initial enzyme concentration. A plot of k_{obs} versus [CPT] was convex, and fitted the empirical equation (eqn. 2):

$$k_{\text{obs.}} = \frac{k_{\text{max.}}[\text{CPT}]}{K + [\text{CPT}]} \quad (2)$$

Values of $k_{\text{max.}}$ and K were determined by a least-squares linear-regression analysis of [CPT]/ $k_{\text{obs.}}$ versus [CPT], for which (from re-arrangement of eqn. 2) the slope is $1/k_{\text{max.}}$ and the intercept at [CPT]/ $k_{\text{obs.}} = 0$ is $-K$ (see Fig. 6). This analysis mode was chosen, by analogy with the extraction of parameters from the Michaelis-Menten equation, as statistically preferable to the double-reciprocal plot. An apparent bimolecular rate constant for CPT inhibition can be obtained from eqn. (2) as the equivalent of k_{Tl} in eqn. (1). For the pig heart

enzyme $k_{\text{Tl}} = k_{\text{max.}}/K$ and has a value of $659 \pm 41 \text{ M}^{-1} \cdot \text{min}^{-1}$, with $k_{\text{max.}} = 0.870 \pm 0.085 \text{ min}^{-1}$ and $K = 1.32 \pm 0.21 \text{ mM}$.

The enzyme from chicken heart is remarkably insensitive to CPT. With CPT concentrations of 0.75 and 1.0 mM (in duplicate) the percentage inhibitions after 20 min were $39 \pm 2\%$ and $45 \pm 5\%$ respectively and the inhibition reaction was still continuing.

Lobster tail lactate dehydrogenase was also inhibited by CPT, and the initial phase of reaction was very rapid. At [CPT] = 0.5 mM approx. 75% of activity was lost in the first 30 s of incubation (at pH 7.4 at 25°C), and the remaining activity decreased to approx. 2% of that of the original enzyme over the next 34 min. Detailed analysis of this system was postponed, as the initial portions of the progress curves for the assays were concave and initial tangents were difficult to draw accurately: the degree of curvature was affected by incubation of the enzyme with CPT.

Irreversibility of CPT inhibition

The reversibility of the inhibition of rabbit muscle lactate dehydrogenase induced by CPT was assessed by incubating enzyme (2 ml; $0.357 \mu\text{M}$) with CPT (0.5 mM) for 10 min at 25°C in pH 7.4

Further support comes from the lack of reversibility of the inhibition by gel filtration over Sephadex G-25.

The nature of the thallium reagent is important. For example, with the rabbit muscle enzyme Ti^+ (as the acetate) was ineffective as an inhibitor, as also was the thallic trifluoroacetate. It appears that specificity is conferred on the reagent by the pendant aryl moieties.

For solubility purposes CPT, with a charged carboxy group, was used for most of these studies in preference to MPT with its neutral side chain. There is some difference in effectivity of these two reagents, but comparison is complicated by the complexity of the time courses (see below). However, it does appear that the negative charge on CPT decreases its overall inhibition efficiency relative to MPT.

With the rabbit muscle enzyme, two phases of modification by CPT are clearly seen (see Fig. 1). Studies in the presence of added cofactor indicate that the faster, primary, phase of the modification is strongly blocked by the presence of NADH, whereas the slower, secondary, phase appears to be unaffected. The presence of NAD^+ did not affect either phase of inhibition.

Additional insight into the modification lies in the results of the reaction of CPT with pig heart lactate dehydrogenase. Again a biphasic inhibition was seen, but now the initial phase was slower than the latter and appeared as a lag. The latter phase (following the first-order rate equation) obeyed saturation kinetics [eqn. (2); see Fig. 6], the easiest explanation of which is an enzyme·CPT complex. Presumably this is formed in the first reaction (i.e. the lag phase), but its effects are still manifest in the kinetics of the final phase. Thus overall we could write eqn. (4) for pig heart lactate dehydrogenase, with phase I slower than phase II:

At present parallel pathways for inhibition cannot be excluded.

In the course of these experiments we noted severe precipitation problems with the procedure. The point at which precipitation occurred depended on the species of lactate dehydrogenase used and its concentration. Higher concentrations of CPT could be used with the heart enzyme than with the rabbit muscle enzyme. When very high lactate dehydrogenase concentrations (approx. 10–20 mg/ml for the rabbit muscle enzyme) were used an immediate white precipitation occurred even at low concentrations (stoichiometric) of CPT. It appears that under these circumstances CPT is

cross-linking the protein. (We attempted to use sodium dodecyl sulphate/polyacrylamide-gel electrophoresis to study this aspect, but were prevented by the insolubility of the precipitated protein under conditions tried to date.)

In principle, amino acid residues cross-linked could be: (1) within the same protein molecule; (2) within the same protein oligomer but on different monomers; (3) on different oligomers. In view of the precipitation at higher protein concentrations, case (3) is likely for lactate dehydrogenase under such conditions. Of course, ArTIL_2 species need not always act to cross-link: in the special environment of a protein it is easy to envisage that only one ligand is displaced by the protein (i.e. ArTIL_2 could behave as univalent modifying agent).

It is possible that with the lower concentrations of lactate dehydrogenase used in the present study CPT is acting bifunctionally, with consecutive trifluoroacetoxy-group replacement corresponding to the phases of eqn. (4). The protection afforded the rabbit muscle enzyme by NADH may indicate that residue Nuc is important to activity. It does not imply that it is at the NADH-binding site. For example, blocking of the cysteine-165 residue of lactate dehydrogenase with several thiol-blocking reagents leads to complete destruction of enzyme activity (Holbrook & Stinson, 1970). X-ray-crystallographic studies have shown that this residue is some 1.0 nm (10 Å) from the substrate-binding site. Inhibition in this case results in the prevention of hydrogen-bonding between substrate and histidine-195, which lies near cysteine-165 (Adams *et al.*, 1973; Holbrook *et al.*, 1975).

The identification of the thalliated residues of the present study remains to be established, but cysteine and histidine residues must be among the prime candidates.

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