Analysis of the mechanism of chloramphenicol acetyltransferase by steady-state kinetics

Evidence for a ternary-complex mechanism

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The mechanism of the enzymic reaction responsible for chloramphenicol resistance in bacteria was examined by steady-state kinetic methods. The forward reaction catalysed by chloramphenicol acetyltransferase leads to inactivation of the antibiotic. Use of alternative acyl donors and acceptors, as well as the natural substrates, has yielded data that favour the view that the reaction proceeds to the formation of a ternary complex by a rapid-equilibrium mechanism wherein the addition of substrates may be random but a preference for acetyl-CoA as the leading substrate can be detected. Chloramphenicol and acetyl-CoA bind independently, but the correlation between directly determined and kinetically derived dissociation constants is imperfect because of an unreliable slope term in the rate equation. The reverse reaction, yielding acetyl-CoA and chloramphenicol, was studied in a coupled assay involving citrate synthase and malate dehydrogenase, and is best described by a rapid-equilibrium mechanism with random addition of substrates. The directly determined dissociation constant for CoA is in agreement with that derived from kinetic measurements under the assumption of an independent-sites model.

The biochemical mechanism of bacterial resistance to chloramphenicol is commonly that of inactivation by *O*-acetylation of the antibiotic, a reaction catalysed by chloramphenicol acetyltransferase (CAT) (EC 2.3.1.28) with acetyl-CoA as the acyl donor (reviewed by Shaw, 1983). The enzyme is cytoplasmic and tetrameric in all bacterial species examined to date, consisting of four identical catalytic subunits, the M_r of which is approx. 25000. The many examples of CAT characterized to date constitute a family of proteins that catalyse the acetylation of chloramphenicol with variable efficiency. The plasmid-associated genes for CAT common to the enteric bacteria specify three main enzyme types (I, II and III), the type III variant

studied in the experiments to be described in the present paper being the most catalytically active. The type I and type III proteins are known to associate with one another to form hybrid tetramers that possess properties of both (Packman & Shaw, 1981*b*), but surprisingly little is known about the catalytic mechanism for either variant. The type III variant of CAT was chosen for a detailed kinetic analysis because of (a) success in obtaining protein crystals of it which were suitable for analysis by X-ray diffraction (Liddell *et al.*, 1978), (b) its catalytic efficiency, and (c) ease and efficiency of purification in high yield by affinity chromatography. Because of extended regions of sequence homology among CAT variants there are good reasons for believing that their catalytic mechanisms will prove to be very similar if not identical. Evidence bearing on the likely importance of a histidine residue in a highly conserved segment of the primary structure has been summarized elsewhere (Shaw, 1983).

Previous studies have demonstrated that the following reactions describe the fate of chloram-

Abbreviations used: CAT, chloramphenicol acetyltransferase; CAT_I, CAT_{II} and CAT_{III}, the three naturally occurring enterobacterial enzyme variants; TSE buffer, 50 mM-Tris/HCl buffer, pH 7.5, containing 100 mM-NaCl and 0.1 mM-EDTA; k_o , the overall catalytic rate constant (also known as k_{cat}).

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phenicol in bacteria that contain CAT (see Shaw, 1983):

Reactions (1) and (3) are both catalysed by CAT, whereas reaction (2) is a non-enzymic and pH-dependent re-arrangement (Nakagawa *et al.*, 1979; Thibault *et al.*, 1980). The enzymic formation of 1,3-diacetoxy chloramphenicol is slow and is not required for inactivation of the antibiotic, since the mono-acetoxy derivatives of chloramphenicol are devoid of activity and fail to bind to ribosomes (Shaw & Unowsky, 1968).

The studies described in the present paper were undertaken to gain a coherent view of the likely mechanism for reaction (1) from the results of a steady-state kinetic analysis of the forward and reverse reactions and to extend previous kinetic observations on the non-enzymic re-arrangement (reaction 2). The symbols and conventions introduced by Dalziel (1957) are used for the steady-state kinetic analysis, wherein substrates A and B in the forward reaction are chloramphenicol and acyl-CoA respectively, and in the reverse reaction P and Q are 3-acetoxy chloramphenicol and CoA respectively.

Materials and methods

Enzymes, reagents and instruments

Type III CAT was purified from cell paste (stored at -20°C) of *Escherichia coli* J53 (R387) obtained from the Microbiological Research Establishment, Porton Down, Wilts., U.K. Citrate synthase, malate dehydrogenase, NAD^+ , NADH and CoA were supplied by the Boehringer Corporation (London) Ltd. [^{14}C]Chloramphenicol and [^3H]CoA were purchased from New England Nuclear. 3-Fluoro chloramphenicol was prepared by Schering (Bloomfield, NJ, U.S.A.). The chloramphenicol analogues were gifts from Parke, Davis (Ann Arbor, MI, U.S.A.). All other reagents were purchased from the Sigma Chemical Co. High-pressure liquid-chromatography experiments were carried out on a Laboratory Data Control System and initial rates measured on Pye-Unicam SP.1800 and SP.8-100 and Perkin-Elmer Lambda 5 spectrophotometers.

Preparation and purification of acyl-CoA analogues

Acyl-CoA analogues were prepared by the method of Simon & Shemin (1953), with the use of acetic anhydride, propionic anhydride and butyric anhydride. The extent of acylation was monitored spectrophotometrically by the reaction of residual CoA with a 1 mM solution of 5,5'-dithiobis-(2-nitrobenzoic acid) in TSE buffer at 25°C . Acylation was judged to be greater than 99% in each case, on the basis of $\epsilon_{412} = 13.6 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Addition of a large excess of chloramphenicol (final concentration 500 μM) and CAT (20–50 units/ml) allowed the concentration of acyl-CoA to be quantified and routinely showed agreement ($\pm 5\%$) with estimates based on the adenine absorbance at 260 nm ($\epsilon_{260} = 15.4 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ in water).

CoA and acyl-CoA analogues were purified by a procedure modified from that of Frey & Utter (1977), with the use of a DEAE-cellulose anion-exchange column with a stepped KCl gradient at pH4 and 4°C . The material was desalted by gel filtration (Sephadex G-10) and eluted in water. Single spots, detected by their absorption of u.v. light, were observed for CoA and each of the acyl-CoA analogues after descending chromatography on Whatman no. 1 paper developed in butanol/acetic acid/water (5:2:3, by vol.).

Preparation and purification of 3-acetoxy chloramphenicol

Acetic anhydride (60 μl , 0.6 mmol) was added in portions to chloramphenicol (194 mg, 0.6 mmol) in pyridine (1 ml) at 4°C . The mixture was warmed to room temperature and extracted with ethyl acetate. The combined organic extracts were dried over MgSO_4 and the solvent was removed by evaporation. The resulting oil was applied to a 120 ml Kieselgel 60 (Merck) silica column developed with chloroform/methanol (99:1, v/v). The eluate was monitored by its absorbance at 278 nm; the diacetoxy derivative of chloramphenicol was found to be eluted in the first column volume and 3-acetoxy chloramphenicol in the second. The identity and purity of 3-acetoxy chloramphenicol was shown by t.l.c. on Kieselgel 60 F_{254} (Merck) silica-gel plates developed in chloroform/methanol (19:1, v/v) (Shaw, 1967). The extent of 1-acetoxy chloramphenicol impurity was less than 1% as demonstrated by high-pressure liquid chromatography (see below).

Determination of the rate of isomerization of 3-acetoxy chloramphenicol to 1-acetoxy chloramphenicol

Duplicate solutions (1 ml) of TSE buffer, acetonitrile (20 mM) and 3-acetoxy chloramphenicol (0.34 mM) were incubated at 25°C and, at the ap-

appropriate times, samples (20 μ l) were analysed for concentrations of monoester by high-pressure liquid chromatography. The isomers were separated at room temperature on a C-18 μ Bondapak reverse-phase column (HPLC Technology Ltd.) eluted with 35% (v/v) acetonitrile in 50 mM-sodium acetate buffer, pH 5.7, at a flow rate of 2 ml/min (Burke *et al.*, 1980). Peaks were identified by absorbance at 278 nm.

CAT standard assay

A modification of the procedure described by Shaw (1975) was used. The standard assay mixture contained TSE buffer, 1 mM-5,5'-dithiobis-(2-nitrobenzoic acid), 0.4 mM-acetyl-CoA and 0.1 mM-chloramphenicol. The reaction was initiated by the addition of enzyme and was monitored by the increase in absorbance at 412 nm. One enzyme unit is defined as that amount of enzyme that catalyses the production of 1 μ mol of product/min at 25°C.

Purification and specific activity of CAT

The type III protein was purified to apparent homogeneity by using an affinity-chromatography method (Zaidenzaig & Shaw, 1976) and the protocol of Packman & Shaw (1981a). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with molecular-mass markers and staining with Coomassie Blue revealed a single protein band with an apparent M_r of 25000. Purified CAT was stored in TSE buffer containing chloramphenicol (0.2 mM) and 2-mercaptoethanol (0.2 mM) at -20°C. The specific activity of purified enzyme, after 18 h dialysis at 4°C against TSE buffer, was found to be 800 ± 50 units/mg, on the basis of enzyme assay under standard conditions and of amino acid analysis and the known amino acid composition of the enzyme (Packman, 1978).

Kinetic measurements

Forward reaction. Measurement of the initial rates of enzymic acetylation of chloramphenicol by acetyl-CoA for kinetic analysis by double-reciprocal plots employed the same conditions as used in the standard assay but over various substrate concentrations (typically in the range $0.3 \times K_m$ to $5 \times K_m$). The concentrations of chloramphenicol and related analogues were determined from absorbance measurements at 278 nm and acyl-CoA compounds as described above.

Reverse reaction. Initial rates of acetylation of CoA by 3-acetoxy chloramphenicol were measured by coupling the CAT reverse reaction with citrate synthase (CS) and malate dehydrogenase (MDH) and monitoring the appearance of NADH at 340 nm, an approach adopted with success by Hersh & Peet (1977) in studies of the mechanism of choline acetyltransferase. In the coupled

system described by eqns. (4)–(6) a fall in the concentration of oxaloacetate is instantaneously reflected in the reduction of NAD⁺ by malate and an increase in absorbance at 340 nm:

Each assay mixture contained TSE buffer (pH 7.5), 5 mM-L-malate, 1.5 mM-NAD⁺, 0.11 mM-NADH, 2 units (μ mol/min) of malate dehydrogenase, 4 units (μ mol/min) of citrate synthase, 3-acetoxy chloramphenicol, CoA and CAT in a final volume of 1 ml. Acetonitrile (final concentration 20 mM) was incorporated to solubilize 3-acetoxy chloramphenicol, and control experiments showed it to have no effect on either the activity of CAT or the coupled assay. None of the components of the coupled assay affected the rate of the CAT forward reaction. Under the above conditions 0.93 equiv. of NADH was produced/equiv. of acetyl-CoA consumed. The stoichiometry is such only if NADH is present in the starting assay mixture (Williamson & Corkey, 1969).

Each double-reciprocal plot was constructed from experimental points resulting from a matrix of four substrate and four coenzyme concentrations, and all reactions were performed in triplicate. For the lowest chloramphenicol and 3-acetoxy chloramphenicol concentrations, in both directions, a maximum of 15–17% depletion of substrate occurred, and linearity of initial rates was observed throughout. Kinetic coefficients in the initial-rate equation:

$$\frac{[E]}{v_0} = \phi_0 + \frac{\phi_A}{[A]} + \frac{\phi_B}{[B]} + \frac{\phi_{AB}}{[A][B]} \quad (7)$$

were calculated from linear intercept and slope replots from manually drawn double-reciprocal plots (Dalziel, 1957).

K_m and k_0 for the hydrolysis of acyl-CoA analogues. Conditions were identical with those of the forward reaction assays except that no chloramphenicol was added and the concentration of CAT (monomer) was typically in the range 0.5–2.0 μ M. Enzyme preparations were exhaustively dialysed against TSE buffer at 4°C before use to remove bound chloramphenicol. All K_m and k_0 values were determined by direct linear plots (Eisenthal & Cornish-Bowden, 1974).

Equilibrium dialysis

Equilibrium dialysis was performed at 26°C in an eight-cell rotating module (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.) essentially as described by Bennett & Shaw (1983). Each cell was divided into two 0.5 ml chambers by a dialysis membrane with a nominal cut-off of M_r 6000–8000. Before use the membrane was boiled in a solution of NaHCO_3 (24 mM) containing EDTA (3.8 mM) and 2-mercaptoethanol (7.5 mM). Membranes were stored at 4°C in 50% (v/v) ethanol. One chamber of each of seven cells received 300 μl of a solution containing CAT in TSE buffer, whereas the opposing chambers contained a range of either [^{14}C]chloramphenicol or [^3H]CoA dilutions in TSE buffer. One cell was used as a control to monitor any loss of enzyme activity during the prolonged incubations (rotation at 10 rev./min; equilibration was complete after 15 h for chloramphenicol and 24 h for CoA). Samples (50 μl) were taken in triplicate from each chamber and placed in 4 ml of either Bray's fluid (for [^{14}C]chloramphenicol) or Fisofluor 1 (for [^3H]CoA) for scintillation counting. The mean values of the ligand present in each chamber were plotted in accordance with Scatchard (1949).

Results

Forward reaction: acetylation of chloramphenicol

Double-reciprocal plots (Lineweaver & Burk, 1934) over a range of fixed substrate concentrations are linear (Fig. 1). Under the experimental conditions used, a small but reproducible ϕ_{AB} term (derived from the slope re-plot) was observed; this is consistent with a sequential mechanism in which ϕ_{AB} is present in the rate equation (eqn. 7) and not a double-displacement mechanism in which ϕ_{AB} is absent (Dalziel, 1957). The incorporation of 3-fluoro chloramphenicol, a competitive inhibitor of CAT ($K_i = 78 \mu\text{M}$), into the assay medium reinforces the conclusion of a sequential mechanism by raising the apparent Michaelis constant for chloramphenicol and therefore the slopes of lines (ϕ_{AB}) on a double-reciprocal plot (Slater, 1955; Koster & Veeger, 1968). The results of such an experiment are recorded in Table 1.

A kinetic approach similar to that used by Dalziel & Dickinson (1966) in studies with liver alcohol dehydrogenase was employed to study the effects of alternative substrates (Fig. 2) on the kinetic coefficients. The hydroxyacetamide and acetamide analogues of chloramphenicol were studied over the concentration ranges 30–500 μM and 13–254 μM respectively, and the results are collected in Table 1. In both of the above cases the acetyl-CoA concentration was varied from 30 to

Fig. 1. Lineweaver-Burk plot for the acetylation of chloramphenicol by chloramphenicol acetyltransferase over a range of acetyl-CoA and fixed chloramphenicol concentrations

The final enzyme (monomer) concentration was 0.14 nM, the chloramphenicol concentrations were 98 μM (1), 19.8 μM (2), 9.9 μM (3) and 6.0 μM (4), and the incubation conditions were pH 7.5 in 0.1 M-Tris/HCl buffer containing 100 mM-NaCl and 0.1 mM-EDTA at 25°C.

R	R'	
COCHCl_2	OH	Chloramphenicol
COCHCl_2	F	3-Fluoro chloramphenicol
COCH_2OH	OH	2-Hydroxyacetamido chloramphenicol
COCH_3	OH	2-Acetamido chloramphenicol

Fig. 2. Chloramphenicol analogues used in this study

380 μM . The corresponding data for propionyl-CoA (38–316 μM) and butyryl-CoA (63–960 μM) are also summarized in Table 1. In the latter experiments the chloramphenicol concentration was varied from 5 to 115 μM .

Reverse reaction: acetylation of CoA

A steady-state kinetic analysis of the reverse reaction needs to take account of the non-enzymic and intramolecular rearrangement of 3-acetoxy chloramphenicol to the 1-acetoxy isomer at alkaline pH (Nakagawa *et al.*, 1979):

To determine the time interval over which reliable initial rates might be observed, the rate for

Table 1. *Kinetic coefficients, at 25°C and pH 7.5, for (i) the forward reaction, acetylation of alternative chloramphenicol substrates and acylation of chloramphenicol by acyl-CoA analogues, and (ii) the reverse reaction*

The kinetic coefficients are those in the initial-rate equation:

$$\frac{[E]}{v_0} = \phi_0 + \frac{\phi_A}{[A]} + \frac{\phi_B}{[B]} + \frac{\phi_{AB}}{[A][B]}$$

where A is chloramphenicol and B is acyl-CoA. For the reverse reaction P is 3-acetoxy chloramphenicol and Q is CoA. ϕ_A/ϕ_0 and ϕ_P/ϕ_0 are the Michaelis constants for chloramphenicol and 3-acetoxy chloramphenicol respectively, and ϕ_B/ϕ_0 and ϕ_Q/ϕ_0 are the Michaelis constants for acyl-CoA and CoA respectively. Each coefficient represents the average for at least two separate experiments.

Substrates		$10^3 \times \phi_0$ (s)	$10^3 \times \phi_A$ ($\mu\text{M} \cdot \text{s}$)	$10^2 \times \phi_B$ ($\mu\text{M} \cdot \text{s}$)	ϕ_{AB} ($\mu\text{M}^2 \cdot \text{s}$)	ϕ_A/ϕ_0 (μM)	ϕ_B/ϕ_0 (μM)	ϕ_{AB}/ϕ_A (μM)	ϕ_{AB}/ϕ_B (μM)
(i)	Acyl acceptor	Acyl donor							
	Chloramphenicol	Acetyl-CoA	2.31 (± 0.03)	26.7 (± 0.3)	17.1 (0.3)	0.63 (± 0.03)	11.6	74.0	23.6
	Chloramphenicol	Acetyl-CoA	1.62 (± 0.18)	99 (± 3.0)	23.1 (± 0.3)	3.15 (± 0.15)			3.7
		(+ 110 μM -3-fluoro chloramphenicol)							
	Hydroxyacetamido chloramphenicol	Acetyl-CoA	2.49 (± 0.15)	225 (± 20.0)	22.8 (± 1.30)	11.88 (± 0.30)	90.4	91.6	52.8
	Acetamido chloramphenicol	Acetyl-CoA	2.46 (± 0.03)	107 (± 9.6)	27.3 (± 0.6)	8.16 (± 0.30)	43.7	111	76.0
	Chloramphenicol	Propionyl-CoA	1.41 (± 0.03)	20.7 (± 0.3)	23.6 (0.6)	0.41 (± 0.02)	14.7	167	19.6
	Chloramphenicol	Butyryl-CoA	4.65 (± 0.15)	104 (± 1.8)	126.0 (± 3.0)	8.22 (± 0.18)	22.3	271	79.2
(ii)	CoA	$10^3 \times \phi_0'$ (s)	$10^3 \times \phi_P$ ($\mu\text{M} \cdot \text{s}$)	$10^2 \times \phi_Q$ ($\mu\text{M} \cdot \text{s}$)	ϕ_{PQ} ($\mu\text{M}^2 \cdot \text{s}$)	ϕ_P/ϕ_0' (μM)	ϕ_Q/ϕ_0' (μM)	ϕ_{PQ}/ϕ_P (μM)	ϕ_{PQ}/ϕ_Q (μM)
	3-Acetoxy chloramphenicol	20.6 $\pm$ (1.4)	270 (± 30)	165 (± 1)	22.6 (± 2.0)	13.1	80.2	83.6	13.7

isomerization was calculated under conditions identical with those used for enzymic assay (pH 7.5 and 25°C) with the use of reverse-phase high-pressure liquid chromatography to measure the concentration of the chloramphenicol esters (see the Materials and methods section). Fig. 3 depicts the results of such an experiment, from which the observed rate constant ($k_{+1} + k_{-1}$) was calculated to be $37 \times 10^{-3} \text{ min}^{-1}$ and the equilibrium constant K_{eq} to be 0.38. Derived values for k_{+1} and k_{-1} were calculated to be $10.4 \times 10^{-3} \text{ min}^{-1}$ and $26.6 \times 10^{-3} \text{ min}^{-1}$ respectively. Since not more than 1% of the 3-acetoxy chloramphenicol might be expected to isomerize during a 1 min incubation, under the conditions employed, calculations of initial rates were limited to this period.

Fig. 3. Isomerization of 3-acetoxy chloramphenicol (3AC) to 1-acetoxy chloramphenicol (1AC) as a function of time (a) The procedure was as described in the Materials and methods section. Concentrations of monoester (●, 3-acetoxy chloramphenicol; ○, 1-acetoxy chloramphenicol) were monitored by the change in peak height after high-pressure liquid chromatography. (b) Determination of the isomerization rate constant by a semi-logarithmic plot. Data are taken from (a).

Double-reciprocal plots for different fixed concentrations of 3-acetoxy chloramphenicol (8–103 μM) and varied concentrations of CoA (32–574 μM) are shown in Fig. 4. Calculated ϕ parameters are given in Table 1. The reverse reaction coefficients are an order of magnitude greater than the corresponding values for the forward reaction and the presence of ϕ_{PQ} is unequivocal. The ratio of the forward and the reverse reaction velocities (v_f/v_r) is 8.8 at pH 7.5 and 25°C.

Reliability of ϕ coefficients

Before analysis of ϕ coefficient relationships for particular mechanisms, the reliability of each parameter was tested by determining the percentage contribution to the overall rate equation (eqn. 7). In nearly every case the coefficients show a major contribution under specified conditions. As an example, for the highest concentrations of chloramphenicol (A) and acetyl-CoA (B) used (98 μM and 380 μM respectively), the percentage contribution of each term to the rate equation is: ϕ_0 , 75%; $\phi_A/[A]$, 9%; $\phi_B/[B]$, 15%; $\phi_{AB}/[A][B]$, 1%. However, ϕ_{AB} values for the combination of chloramphenicol with acetyl-CoA or propionyl-CoA do not contribute significantly and are therefore likely to be inherently less reliable than other parameters. Hence minimum emphasis has been placed on these values and parameter ratios that contain them.

Fig. 4. Lineweaver-Burk plot for the acetylation of CoA by CAT over a range of 3-acetoxy chloramphenicol and fixed CoA concentrations

(a) The final enzyme (monomer) concentration was 1.37 nM, the CoA concentrations were 574 μM (1), 106 μM (2), 52 μM (3) and 32 μM (4), and the conditions were as described in the Materials and methods section.

Estimation of binding constants

Ligand-binding studies for the forward-reaction and reverse-reaction substrates were undertaken in order to compare directly obtained binding constants with the corresponding values derived from steady-state kinetic analysis. 3-Acetoxy chloramphenicol was not studied, owing to the pH-dependent isomerization.

The binding of chloramphenicol and CoA by CAT were studied by equilibrium dialysis at 26°C

in the same buffer system used for the initial-rate determinations. The results, depicted in Figs. 5(a) and 5(b), illustrate the binding of these substrates by CAT, in the absence of the second substrate, with a 1:1 stoichiometry (per monomer). The calculated dissociation constants were 11.5 (±0.1) μM for chloramphenicol and 78 (±2) μM for CoA.

A direct estimation of the binding constant for acetyl-CoA by substrate-depletion techniques [equilibrium dialysis, Hummel & Dryer (1962) gel filtration, and flow dialysis (Colowick & Womack, 1969)] was made impossible by the low but significant intrinsic thioesterase activity associated with CAT_{III}. The hydrolysis of acetyl-CoA by substrate concentrations of CAT in the absence of chloramphenicol was first noted by Zaidenzaig & Shaw (1978) for the type I variant of the enzyme, but the phenomenon was not examined in detail. The apparent *K_m* for hydrolysis of the three acyl-CoA analogues are presented in Table 2. The catalytic constant (*k₀*), in each case, was 0.01–0.02% of that observed for each of the corresponding acylation reactions.

Discussion

Evidence for a sequential mechanism

The presence and subsequent amplification of φ_{AB} by the incorporation of a competitive inhibitor (3-fluoro chloramphenicol) in the forward reaction is consistent with a ternary-complex mechanism. The corresponding terms for each of the chloramphenicol analogues, and that of the reverse reaction (φ_{PQ}), are considerably greater and must therefore contribute to the overall rate equation. Zaidenzaig & Shaw (1978) noted the absence of partial reactions between acetyl-CoA and CoA when they were incubated with the type I variant of CAT, an observation in accord with the steady-state results now presented for the type III enzyme. The formation of a ternary complex, between enzyme and substrates, may proceed by a variety of pathways, as outlined in Scheme 1. The present investigation attempted to distinguish between these possibilities.

Fig. 5. Analysis of the binding of (a) [¹⁴C]chloramphenicol and (b) [³H]CoA to the type III variant of CAT by equilibrium dialysis

The procedure was as described in the Materials and methods section and the results were analysed in accordance with Scatchard (1949). For these experiments the concentration of enzyme monomer was 15–30 μM.

Table 2. Comparison of apparent *K_s* values (φ_{AB}/φ_A) for acyl-CoA analogues, derived from the data in Table 1, with the corresponding Michaelis constants for the enzyme-catalysed hydrolysis

	Estimates in parentheses represent unreliable values.	
	<i>K_s</i> (app.) (μM)	<i>K_m</i> (app.) (μM)
Acetyl-CoA	(23.6), 52–76	68
Propionyl-CoA	(19.6)	32
Butyryl-CoA	79	65

Scheme 1. Basic representation of the possible orders of binding of substrates and products to CAT

Key: A, chloramphenicol; B, acetyl-CoA; P, 3-acetoxy chloramphenicol; Q, CoA. Deletion of the lower (or alternatively the upper) pathway yields a compulsory-order mechanism, whereas the rapid equilibration of the two substrates and both products with the enzyme leads to a special case of the random-order mechanism.

Mechanistic interpretations derived from the use of alternative acyl acceptors and donors

When compared with the natural substrate, both chloramphenicol analogues yielded no change in ϕ_0 , a small increase in ϕ_B and substantial increases in both ϕ_A and ϕ_{AB} . The constancy of ϕ_0 indicates a common rate-determining step for the acetylation of alternative substrates, a result observed with horse liver alcohol dehydrogenase and interpreted by Dalziel & Dickinson (1966) as evidence for co-enzyme dissociation as the rate-determining step. Furthermore, if the forward reaction, catalysed by CAT, were a compulsory-order mechanism in which B leads, then ϕ_B would be equivalent to the association rate constant and should not vary in spite of alterations to the structure of substrate A. The relatively slight variation in ϕ_B (in comparison with ϕ_A) for the chloramphenicol analogues suggests that acetyl-CoA may lead in a compulsory-order mechanism. The latter condition requires ϕ_{AB}/ϕ_A to be the true dissociation constant (Dalziel & Dickinson, 1966) for acetyl-CoA and should remain constant. The derived values for ϕ_{AB}/ϕ_A (Table 1) appear to range from $24 \mu\text{M}$ to $76 \mu\text{M}$; however, the inherent unreliability of the ϕ_{AB} value determined for the parent substrates (see the Results section) suggests that more confidence should be placed on the corresponding values of ϕ_{AB}/ϕ_A for the chloramphenicol analogues (53 and $76 \mu\text{M}$). Subsequent studies (see below and Table 2) on the hydrolysis of acetyl-CoA catalysed by CAT yielded an apparent K_m (and K_s) for acetyl-CoA of $68 \mu\text{M}$, a value in keeping with the derived K_s (ϕ_{AB}/ϕ_A) from the data for *N*-acyl analogues of chloramphenicol.

Table 3. Specificity of CAT for acyl-CoA analogues of various acyl-chain lengths
Data are taken from Table 1.

	k_0 (s^{-1})	K_m (μM)	k_0/K_m ($\text{M}^{-1}\cdot\text{s}^{-1}$)
Acetyl-CoA	433	74	0.59×10^7
Propionyl-CoA	709	167	0.42×10^7
Butyryl-CoA	215	271	0.08×10^7

Returning to ϕ_B , we should note that the narrow range of values for this term may reflect subtle effects of variations in the structure of the ligand occupying the chloramphenicol site on the state of acetyl-CoA in the ternary complex.

An increase in length of the acyl-donor side chain by the addition of an extra methylene group (propionyl-CoA) leads to a small decrease in ϕ_0 relative to acetyl-CoA, whereas ϕ_A and ϕ_B are relatively unaffected. Although a decrease in ϕ_0 shows propionyl-CoA to be turned over at a greater rate than acetyl-CoA, the concomitant increase in K_m yields an efficiency quotient (k_0/K_m^B) that is essentially the same for both acyl donors (Table 3). The appreciable increases (as compared with results for acetyl-CoA) that are observed in all ϕ coefficients when butyryl-CoA is the acyl-donor show it to be a significantly poorer substrate than acetyl-CoA and propionyl-CoA, an observation also reflected in the corresponding k_0/K_m^B values (Table 3). It is apparent that the catalytic constant is sensitive to changes in acyl-chain length and that the latter must affect the rate-determining step. This observation contrasts with the chloramphenicol analogue data, which suggest that CoA dissociation may be rate-determining. Two possible explanations to resolve these apparently conflicting results are the following. (i) Both CoA dissociation and interconversion of the ternary complex may contribute to the rate-determining step (see, for example, the case of choline acetyltransferase; Hersh, 1982). (ii) CoA dissociation alone may be rate-determining when acetyl-CoA and propionyl-CoA are substrates, but this situation breaks down for butyryl-CoA, and the turnover of the ternary complex becomes rate-limiting. A similar situation obtains for liver alcohol dehydrogenase (Dalziel & Dickinson, 1966), where the rate-determining step for the reaction shifts from coenzyme dissociation in the reduction of primary alcohols to ternary-complex interconversion for secondary alcohols.

Reverse reaction

For a rapid-equilibrium random-order mechanism, substrate K_s values may be derived from ϕ ratios and, if the two substrates do not affect one another's binding, $\phi_{PQ}/\phi_Q = \phi_P/\phi_0'$ and likewise

$\phi_{PQ}/\phi_P = \phi_Q/\phi_0'$. These relations are clearly observed for the reverse reaction, with close agreement between the Michaelis constants for the substrates and their kinetically derived dissociation constants. This is further substantiated by the near-identical values for K_{CoA}^{CoA} ($78\ \mu\text{M}$), as determined by equilibrium dialysis, and ϕ_{PQ}/ϕ_P ($84\ \mu\text{M}$) from kinetic data. Re-arrangement of the above ϕ coefficient relations leads to the equality $\phi_0' = \phi_P \cdot \phi_Q / \phi_{PQ}$ (Dalziel, 1957), which is also fulfilled (wherein the calculated value for $\phi_P \cdot \phi_Q / \phi_{PQ}$ is 0.02 s and that for ϕ_0' is 0.021 s ; see Table 1). Such an outcome is indicative of the special case of the rapid-equilibrium random-order mechanism in which there is no substrate synergism.

Substrate dissociation constants

The apparent agreement of K_m and K_s for the substrates in the reverse reaction prompted a study of the binding of chloramphenicol and acetyl-CoA to the enzyme. The dissociation constant for chloramphenicol ($11.5\ \mu\text{M}$), as determined by equilibrium dialysis, does not agree with the predicted value ($3.7\ \mu\text{M}$) from the steady-state studies (assuming an independent-sites model), but the unreliability of ϕ_{AB} precludes any meaningful comparison. However, we may note the close agreement of K_s for chloramphenicol with the derived Michaelis constant ($\phi_A/\phi_0 = 11.6\ \mu\text{M}$)

The intrinsic thioesterase activity of CAT indirectly leads to an estimate of K_s for the three acyl-CoA analogues in the form of Michaelis constants for hydrolysis (see Table 2). The K_m for acetyl-CoA hydrolysis ($68\ \mu\text{M}$) lies within the predicted range of reliable ϕ_{AB}/ϕ_A values derived from steady-state studies with the hydroxyacetamide and acetamide analogues of chloramphenicol ($52\text{--}76\ \mu\text{M}$). The low value for the apparent dissociation constant for propionyl-CoA ($19.6\ \mu\text{M}$) is mirrored in the observed K_m for its hydrolysis ($32\ \mu\text{M}$), which is below those of the other acyl-CoA compounds. Since the K_m for butyryl-CoA hydrolysis ($65\ \mu\text{M}$) also approaches the predicted K_s ($79\ \mu\text{M}$), there is a case for considering the Michaelis constant for hydrolysis of the acyl-CoA analogues by CAT as a first approximation of their dissociation constants. A basic kinetic scheme for a single substrate is proposed in which it is assumed that k_{+2} is the rate-limiting step for the overall reaction. Given that k_{+2} is 0.1 s^{-1} (0.02% of the rate of chloramphenicol acetylation) and that coenzyme dissociation rate constants are normally in the range of $10\text{--}10^3\text{ s}^{-1}$ (Fersht, 1977), it is reasonable to assume that $k_{+2} \ll k_{-1}$ and therefore that K_m reduces to K_s for hydrolysis.

The above analysis is based on the premise that the enzymic hydrolysis of the acyl thioesters is due to CAT rather than a contaminating thioesterase

that becomes kinetically significant at high concentrations of added enzyme. The latter view seems untenable on the basis of two independent observations. First, the CAT and thioesterase activities have been observed to co-purify to apparent homogeneity with conventional (non-affinity) and affinity purification methods (results not shown). Secondly, covalent and stoichiometric modification of highly purified CAT by 3-bromo-acetoxy chloramphenicol is accompanied by parallel inhibition of both the acetylation and the hydrolysis reactions (C. Kleanthous & W. V. Shaw, unpublished work).

The intrinsic hydrolytic activity of CAT bears a superficial resemblance to that of another enzymic group-transfer reaction, the ATPase activity of hexokinase (Trayser & Colowick, 1961). The rate of hydrolysis of ATP was 0.003% that of the phosphorylation rate and K_m^{ATP} for hydrolysis was 40-fold higher than that observed for the hexokinase reaction. Dela Fuente *et al.* (1970) noted that lyxose, an inert glucose analogue, promoted the hydrolytic activity of hexokinase and decreased the K_m^{ATP} to a value equal to that obtained for phosphorylation of glucose. The result was interpreted as showing substrate synergism between ATP and lyxose, the latter binding first. In the case of CAT, however, the Michaelis constant for acetyl-CoA hydrolysis approximates that observed for acetylation. Moreover, the addition of the substrate analogue 3-fluoro chloramphenicol had no effect on the observed K_m for thioester hydrolysis (results not shown). Such observations are consistent with the notion of independent binding of substrates.

Concluding remarks

The data presented strongly favour a ternary-complex mechanism for chloramphenicol acetyltransferase. The rate-determining step for CAT appears to be affected by acyl-chain length but not by the identity of the acyl-acceptor. If the interconversion of the ternary complex contributes significantly to the rate-limiting step, then the tightness of chloramphenicol binding is not a contributing factor. Ligand-binding studies for the forward reaction clearly show that both substrates bind independently of each other. Furthermore, the binding constants for chloramphenicol and acetyl-CoA (11.5 and $68\ \mu\text{M}$ respectively) show good agreement with their respective Michaelis constants (11.6 and $74\ \mu\text{M}$). This situation is apparently mirrored in the reverse reaction, where the kinetically derived values of K_m and K_s , for both substrates, match very closely, and K_{CoA}^{CoA} ($78\ \mu\text{M}$), as determined by equilibrium dialysis, agrees well with the derived value ($\phi_{PQ}/\phi_P = 84\ \mu\text{M}$), assuming an independent-sites model. However, the use of different acyl-acceptors, in the forward reaction, has led to the

suggestion that acetyl-CoA binds before chloramphenicol.

Andersson *et al.* (1984) have reconciled similarly conflicting observations for liver alcohol dehydrogenase. Both substrates bind independently of one another, but, under the conditions normally chosen for kinetic analysis, the E-NADH pathway to the ternary complex is preferred. Reaction flow by this route is due to a decreased rate of coenzyme dissociation from the ternary complex as compared with that observed for the aldehyde. Their investigation highlights the limitations of rate equations in describing the order of ligand binding for ternary-complex systems when the individual association and dissociation rate constants are not known. Therefore, although a preferred-order pathway for the forward reaction seems a likely conclusion for CAT_{III}, this may not obtain for all concentrations of chloramphenicol or reaction conditions different from those employed in the present study.

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