Substrate Binding to Chloramphenicol Acetyltransferase: Evidence for Negative Cooperativity from Equilibrium and Kinetic Constants for Binary and Ternary Complexes†

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ABSTRACT: Chloramphenicol acetyltransferase (CAT) catalyzes the acetyl-CoA-dependent acetylation of chloramphenicol by a ternary complex mechanism with a rapid equilibrium and essentially random order of addition of substrates. Such a kinetic mechanism for a two-substrate reaction provides an opportunity to compare the affinity of enzyme for each substrate in the binary complexes ($1/K_d$) with corresponding values ($1/K_m$) for affinities in the ternary complex where any effect of the other substrate should be manifest. The pursuit of such information for CAT involved the use of four independent methods to determine the dissociation constant (K_d) for chloramphenicol in the binary complex, techniques which included stopped-flow measurements of on and off rates, and a novel fluorometric titration method. The binary complex dissociation constant (K_d) for acetyl-CoA was measured by fluorescence enhancement and steady-state kinetic analysis. The ternary complex dissociation constant (K_m) for each substrate (in the presence of the other) was determined by kinetic and fluorometric methods, using CoA or ethyl-CoA to form nonproductive ternary complexes. The results demonstrate an unequivocal decrease in affinity of CAT for each of its substrates on progression from the binary to the ternary complex, a phenomenon most economically described as negative cooperativity. The binary complex dissociation constants (K_d) for chloramphenicol and acetyl-CoA are 4 μM and 30 μM whereas the corresponding dissociation constants in the ternary complex (K_m) are 12 μM and 90 μM, respectively. Reasoning from the known binary complex structures, the 3-fold decrease in affinity of CAT for each of its substrates en route to the tetrahedral intermediate and the transition state may be a necessary prerequisite for their formation, probably involving subtle but important changes in the structure of the enzyme and/or the conformations of bound substrates.

The antibiotic chloramphenicol effectively inhibits protein synthesis by binding to the peptidyltransferase center of prokaryotic ribosomes [reviewed in Gale et al. (1981)]. Bacterial resistance is most commonly conferred by 3-O-acetylation of chloramphenicol, utilizing acetyl-CoA as acyl donor, a reaction catalyzed by chloramphenicol acetyltransferase (CAT;[1] EC 2.3.1.28; Shaw, 1967; Suzuki & Okamoto, 1967). The acetylated antibiotic is unable to bind to ribosomes and is devoid of antimicrobial activity (Shaw & Unowsky, 1968).

CAT activity has been detected in a wide range of chloramphenicol-resistant bacteria [reviewed by Shaw (1983); Shaw & Leslie, 1991]. Of the 11 naturally occurring CAT variants for which nucleotide sequences have been determined, the type III enzyme has been the focus of structural and mechanistic studies, mainly because the structures of the binary complexes of CAT_{III} and its substrates have been determined. The complex with chloramphenicol has been determined at 1.75-Å resolution (Leslie, 1990), and the CAT·CoA complex is known at 2.4 Å (Leslie et al., 1988).

CAT_{III} is a trimeric enzyme of identical subunits of M_r = 25 000 with three active sites located at the subunit interfaces. Residues comprising the major portion of both the chloramphenicol and CoA binding sites are supplied by one subunit, whereas a catalytically essential histidine residue (His-195)[2] is provided by the opposing subunit. The two binding sites together form a remarkable tunnel, approximately 25 Å long, extending through the protein and occupied by solvent in the absence of substrates (Leslie et al., 1988). Chloramphenicol and acetyl-CoA approach the deeply buried active site from opposite ends of the tunnel to bring the 3-hydroxyl of chloramphenicol and the S atom of CoA within 2.8 and 3.3 Å, respectively, of the $N^{\epsilon 2}$ atom of His-195. The structural separation and independence of the binding sites for acyl donor and acceptor were predicted by the results of steady-state kinetic studies implying a sequential (ternary complex) mechanism for CAT_{III} with a rapid equilibrium and essentially random order of addition of substrates (Figure 1). In the absence of chloramphenicol, CAT hydrolyzes acetyl-CoA at a rate 10^3-fold lower than that of the transacetylation reaction (Kleanthous & Shaw, 1984).

The likely importance of His-195 in the catalytic mechanism of CAT_{III} was first inferred from the results of studies with 3-(bromoacetyl)chloramphenicol, an active site directed inhibitor which inactivates CAT_{III} through a specific stoichiometric alkylation of the $N^{\epsilon 2}$ of His-195 (Kleanthous et al., 1985). The proposed reaction mechanism assigns a general base role to the $N^{\epsilon 2}$ of His-195; the latter deprotonates the 3-hydroxyl of chloramphenicol, promoting nucleophilic attack of the oxygen at the carbonyl of the thioester of acetyl-CoA (Kleanthous et al., 1985). The resulting tetrahedral intermediate is probably stabilized in the transition state by a

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[1] Abbreviations: CAT, chloramphenicol acetyltransferase; CAT_{III}, type III variant of CAT; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); *p*-cyano-CM, D-*threo*-1-(4-cyanophenyl)-2-(dichloroacetamido)-1,3-propanediol; *p*-iodo-CM, D-*threo*-1-(4-iodophenyl)-2-(dichloroacetamido)-1,3-propanediol; TSE buffer, 50 mM Tris-HCl buffer, pH 7.5, containing 100 mM NaCl and 0.1 mM EDTA.

[2] Alignment of the amino acid sequences of CAT variants (Shaw & Leslie, 1991) has resulted in a general numbering system which is used here.

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hydrogen bond between its oxyanion and the hydroxyl of Ser-148 (Lewendon et al., 1990).

Studies with CAT_{III} to date have not addressed the possibility of substrate cooperativity[3] within the system. Steady-state kinetic analysis suggested that the binding of each substrate might decrease the affinity of the enzyme for the other prior to the catalytic step (Kleanthous & Shaw, 1984), although in the absence of a structure of the ternary complex of CAT_{III} with appropriate substrates (or nonreactive analogues) the basis of such negative cooperativity remained unclear. More recently, it has become possible to monitor the formation of binary and (nonproductive) ternary complexes of CAT_{III} and substrates (or inhibitors) by means of changes in protein fluorescence. In particular, the assignment of the contribution of each of the three tryptophans of CAT_{III} to intrinsic fluorescence, and to the changes which occur on substrate binding, has led to the development of sensitive and precise assays of ligand binding to the chloramphenicol and CoA sites (Ellis et al., 1991). Such an approach has been particularly valuable in measuring the dissociation constant of the CAT·acetyl-CoA complex which is not accessible by equilibrium dialysis because of the thioesterase activity of CAT_{III} in the absence of an acyl acceptor. We report here the dissociation constants of each of the substrates of CAT_{III} from their respective binary complexes, and from a number of nonproductive ternary complexes. The equilibrium binding data for chloramphenicol are supported by an investigation of the kinetics of its binding using the stopped-flow technique. Together the results support the notion of negative cooperativity in the binding of both chloramphenicol and acetyl-CoA to CAT_{III} whereby the association constant for enzyme and each substrate in the ternary complex, prior to the formation of the tetrahedral intermediate, and ultimately that of the transition state, is lower than in the corresponding binary complexes. The results are discussed with references to the free energy of the system as a whole, in moving from the ground state(s) to the transition state, and the likelihood of "strain" in the structure of the ternary complex.

Experimental Procedures

Purification of CAT. CAT was purified from *Escherichia coli* extracts by affinity chromatography on chloramphenicol–Sepharose as previously described (Lewendon et al., 1988). Chloramphenicol used as eluent in the previous step was removed by gel-permeation chromatography on Sephadex G-50. The purity of enzyme preparations was assessed by SDS–polyacrylamide gel electrophoresis where a single band was always observed with loadings of up to 50 μg of protein per track. The concentration of purified CAT was calculated from the $\epsilon^{1\%}_{280} = 13.1$ or by the method of Lowry (1951), standardized by amino acid analysis.

Assay of CAT Activity. CAT activity was assayed spectrophotometrically at 25 °C using a modification of the procedure described by Shaw (1975). The standard assay mixture contained TSE buffer, pH 7.5, 1 mM 5,5′-dithiobis(2-nitrobenzoic acid), 0.1 mM chloramphenicol, and 0.4 mM acetyl-CoA. The reaction was initiated by addition of enzyme, and the rate of formation of CoA was monitored by its reaction with DTNB and liberation of the thionitrobenzoate dianion ($\epsilon_{412nm} = 13\,600\ M^{-1}\ cm^{-1}$). One unit of enzyme activity is defined as the amount converting 1 μmol of substrate to product per minute under the standard assay conditions.

In the steady-state kinetic analysis of the forward transacetylation reaction concentrations of chloramphenicol and acetyl-CoA were varied in the standard assay mixture. Linear initial rates were measured over times during which less than 15% depletion of substrates occurred. Kinetic parameters were determined as described previously (Kleanthous & Shaw, 1984).

Equilibrium Dialysis. Equilibrium dialysis was performed at 25 °C in three eight-cell rotating modules (Hoefer Scientific Instruments, San Francisco, CA) essentially as described by Kleanthous and Shaw (1984). Each cell was divided into two 0.5-mL chambers by a M_r = 6000–8000 cutoff dialysis membrane. Initially, one chamber of each cell contained 300 μL of CAT in TSE buffer, pH 7.5, whereas the opposing chambers contained a range of concentrations of *N*-([1-^{14}C]dichloroacetyl)chloramphenicol (New England Nuclear) in TSE buffer. A control cell was used to monitor any loss of enzymic activity during the 24-h equilibration period. Samples (50 μL) were taken in triplicate from each chamber and assayed for ^{14}C content by scintillation counting. The mean values of the ligand present in each chamber were plotted both directly as [bound] versus [free] and in accordance with Scatchard (1949) and the data were analyzed by the program Enzfitter (Leatherbarrow, 1987).

Fluorometric Titration. Fluorometric titrations were carried out by both manual addition of substrate and automatic addition with a programmable syringe drive (Harvard Pump 22). Typically, 3 mL of CAT_{III} (1 μM) in TSE buffer, pH 7.5, was used for each titration. Control titrations were also performed containing the amount of free tryptophan required to give similar fluorescence intensity to that of the CAT_{III} sample. All titrations were carried out at 25 °C and considered complete when no further intensity changes were noted on addition of more substrate.

Manual titrations were performed in a Perkin-Elmer LS-5B luminescence spectrometer, using a response time of 3 s and excitation and emission slit widths of 2.5 nm. Tryptophan fluorescence was selected, and substrate inner filer effects were minimized, by excitation at 295 nm. Emission was monitored at 335 nm. Sequential additions, using a Hamilton syringe, of small volumes of ligand were made from concentrated stock solutions, and after thorough mixing, the new fluorescence intensity of the sample was measured in arbitrary units. The fluorometer output was relayed directly to a Perkin-Elmer GP 100 plotter for a hard copy of the data.

Automated titrations were performed using a custom-built apparatus with the optical components described previously (Jackson & Bagshaw, 1988). Excitation was achieved using the 297-nm line from a 200-W Hg lamp, and emission was detected at 350 nm by means of UG11 and WG335 filters in front of the photomultiplier. In addition, the transmitted light was monitored using a Hamamatsu S1336 photodiode to provide an upper limit for the extent of the inner filter effect. The titrant was added from a 250-μL Hamilton syringe, driven by an infusion pump (Harvard Apparatus, Edenbridge, Kent, U.K.), via fine-bore Teflon tubing and a stainless steel needle which terminates in a 0.3-mm-diameter Teflon tip. This diameter was necessary to minimize diffusion of the titrant between additions. The contents of the cuvette were stirred by an overhead motor. The pump, stirrer, and fluorometer were all under the control of an Apple II+ computer allowing "intelligent" operation. At the start of the titration the physical parameters of the system and concentration of the stock titrant

[3] We use the term cooperativity to signify a joint action or effect not observed in the binding of either substrate to the enzyme in the absence of the other. In this context, a measure of antagonism or *negative* cooperativity is the decrease in binding constant on association of the second ligand, without implying a mechanism for the observed effect.

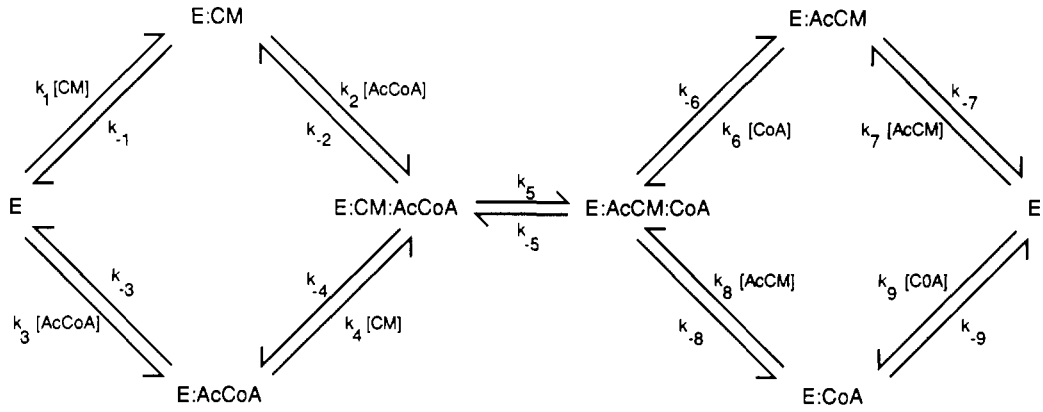


FIGURE 1: The kinetic mechanism of CAT_{III}. Key: CM, chloramphenicol; AcCM, 3-acetylchloramphenicol; AcCoA, acetyl-CoA.

were entered, together with the final titrant concentration required. Initially the pump dispensed 0.1 μL of titrant to the cuvette which was then stirred for a preset time (typically 2 s), and the fluorescence signal, after it had stabilized, was logged using a 12-bit A/D converter. Depending on the extent of the fluorescence change relative to a present value, the pump would double or halve the previous volume added so as to track the titration curve with optimum spacing. Typically, the titration parameters were arranged so that between 25 and 100 additions were made. The apparatus performed titrations satisfactorily in this intelligent mode when the total fluorescence change was as little as 4%. The titrator could also be operated in the menu-driven mode, where additions were made according to a predetermined instruction set.

Data were plotted as fluorescence intensity versus substrate concentration and fitted to a hyperbolic expression when $K_d \gg$ [enzyme], otherwise to a quadratic function, by a nonlinear regression analysis program (Marquadt algorithm) to calculate the dissociation constant of the system.

Stopped-Flow Fluorometry. A SF-17MV stopped-flow spectrometer (Applied Photophysics, Leatherhead, U.K.), with a dead time of 2 ms, was used for the measurement of association and dissociation rate constants. The light source was a 150-W xenon lamp, 295 nm was selected as excitation wavelength, and a 1-mm slit width was used. The drive syringes were driven by a pneumatic actuator operated by compressed nitrogen (pressure from 5 to 8 bar). The temperature of the drive syringes and observation chamber was controlled by water circulation. Stock solutions of ligands and enzyme were diluted to the desired concentrations with degassed TSE buffer, pH 7.5, immediately prior to loading into the drive syringes. Typically, 10 traces were collected to be signal-averaged and saved on disc. Data acquisition and processing was controlled by a 32-bit processor Archimedes workstation, which was also used for subsequent nonlinear regression analysis of the averaged traces.

Association rate constants were calculated by measurement of reaction rates, at low enzyme concentration, over a range of substrate concentrations, under pseudo-first-order conditions. The resultant data were plotted according to the equation $k_{obs} = k_1[S] + k_{-1}$ for a one-step mechanism, yielding the association (k_1) and dissociation (k_{-1}) rate constants by linear regression analysis. In addition, dissociation rate constants were found from the displacement of substrate with a suitable analogue.

RESULTS

Binding of Chloramphenicol in the Binary Complex with CAT_{III}. The steady-state kinetic analysis of a two-substrate rapid equilibrium random-order enzymic mechanism yields two dissociation constants, K_d and K_m, for each substrate where K_d is the dissociation constant for the binary complex with the protein alone and K_m is the dissociation constant for the ternary complex, with the second substrate bound (Figure 1). K_d and K_m are equal when each substrate has no effect on the binding of the other (Engel, 1985).

In the case of CAT_{III}, such a kinetic analysis reveals a 3-fold difference in affinity for chloramphenicol in the binary (K_d = 4 μM) as compared to the ternary (K_m = 12 μM) complex (Tables I and III). Nonetheless, earlier ligand binding studies by equilibrium dialysis failed to support such apparent substrate synergism, yielding a K_d value for chloramphenicol (11.5 μM) approximating K_m (Kleanthous & Shaw, 1984). As the latter value was measured directly, it was deemed more reliable than that derived from steady-state kinetic parameters, with

Table I: Comparison of Dissociation Constants for Binary Complexes of CAT and Substrates Obtained by Different Techniques[a]

	K_d (μM)			
substrate	steady-state derivation	fluorometric titration	equilibrium dialysis	stopped-flow rate measurements
chloramphenicol	4.0	4.3	3.6*	4.1
p-cyano-CM	3.2	2.1	ND	ND
acetyl-CoA	31	28	ND	ND

[a] An asterisk indicates a high-affinity site; see text for discussion. ND = not determined.

Table II: Comparison of Dissociation Constants for Acetyl-CoA in Binary and Ternary Complexes with CAT

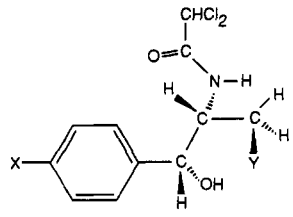
complex	K_d (acetyl-CoA) (μM)
CAT·acetyl-CoA	28[a,b]
CAT·chloramphenicol·acetyl-CoA	90[b]
CAT·3-deoxy-CM·CoA	26[a]
CAT·3-acetyl-CM·acetyl-CoA	102[a]
CAT·3-iodo-CM·acetyl CoA	61[a]

[a] Dissociation constants measured by fluorometric titration as described under Experimental Procedures. [b] Dissociation constant measured by steady-state kinetic analysis as described under Experimental Procedures.

Table III. Rate Constants for Association and Dissociation of Chloramphenicol from CAT

complex	k_1 (M^{-1} s^{-1})	k_{-1} (s^{-1})	K_d (μM)
CAT·chloramphenicol	4.8×10^7	207	4.3[a]
CAT·CoA·chloramphenicol	4.7×10^7	194	3.9[a]
CAT·ethyl-CoA·chloramphenicol	4.8×10^7	608	12.4[a]
CAT·acetyl-CoA·chloramphenicol	ND	ND	12.0[b]

[a] Equilibrium dissociation constant for CM calculated from measured rate constants as described under Experimental Procedures and Results. [b] Parameters (K_m) measured by steady-state kinetic analysis as described under Experimental Procedures.



X	Y	Compound
$-NO_2$	-OH	Chloramphenicol
-CN	-OH	p-cyano-CM
$-NO_2$	-H	3-deoxy-CM
$-NO_2$	-OAc	3-acetyl-CM
$-NO_2$	-I	3-iodo-CM

FIGURE 2: Structure of chloramphenicol and its analogues used in the present study. All compounds were of the D-*threo* configuration.

the tentative conclusion that $K_d = K_m$ for chloramphenicol and that the reaction proceeded without substrate synergism. The recognition that changes in the intrinsic fluorescence of CAT_{III} occur on binding its substrates (Ellis et al., 1991) prompted a reassessment of the problem and the development of an alternative quantitative method to resolve the kinetic anomaly.

Upon binding, chloramphenicol reduces the intrinsic tryptophan fluorescence of CAT_{III} by 29% (Ellis et al., 1991). However, since the quench is dominated by the inner filter effect of chloramphenicol (λ_{max} = 278 nm), it cannot be used to quantitate the extent of saturation of CAT_{III} in a direct titration with chloramphenicol. However, an analogue of the natural substrate, *p*-cyano-CM (λ_{max} = 234 nm) in which the cyano substituent replaces the nitro group of the phenyl ring of chloramphenicol (Figure 2), was found to produce neither changes in protein fluorescence nor an inner filter effect. Since the analogue is also readily acetylated by CAT_{III} (Ellis et al., 1991), it seemed an ideal tool for use in a competition binding assay from which dissociation constants for both acyl acceptors could readily be determined.

The procedure involves carrying out a series of titrations with *p*-cyano-CM in the presence of varying known concentrations of chloramphenicol. Competition between the two substrates for the binding sites of CAT_{III} leads to displacement of the prebound chloramphenicol from the binary complex with a corresponding "dequench" of tryptophan-related fluorescence. The total amplitude change observed in each titration was plotted against the concentration of chloramphenicol to yield a hyperbola from which nonlinear regression analysis provides the K_d for chloramphenicol (Figure 3a). Furthermore, the apparent K_d values arising from the primary data may be plotted according to the equation

$$K_d^{app} = K_d^A(1 + [B]/K_d^B)$$

where A = *p*-cyano-CM and B = choramphenicol, to yield K_d values for both acyl acceptors (Figure 3b). In this way, dissociation constants were found to be 4.3 ± 0.3 μM and 2.1 ± 0.2 μM for chloramphenicol and *p*-cyano-CM, respectively, values in agreement with those found from steady-state kinetic analysis.

Since the inner filter effect of chloramphenicol is instantaneous ($\sim 10^{-15}$ s) compared to the millisecond time scale of the quench due to the binding of chloramphenicol at low concentrations, it does not hamper time-resolved fluorescence monitoring of binding. Stopped-flow fluorometry may be used, therefore, to measure chloramphenicol dissociation and association rate constants directly.

In determining the association rate constant, it is necessary to satisfy the requirement [E] ≪ [S] for a pseudo-first-order

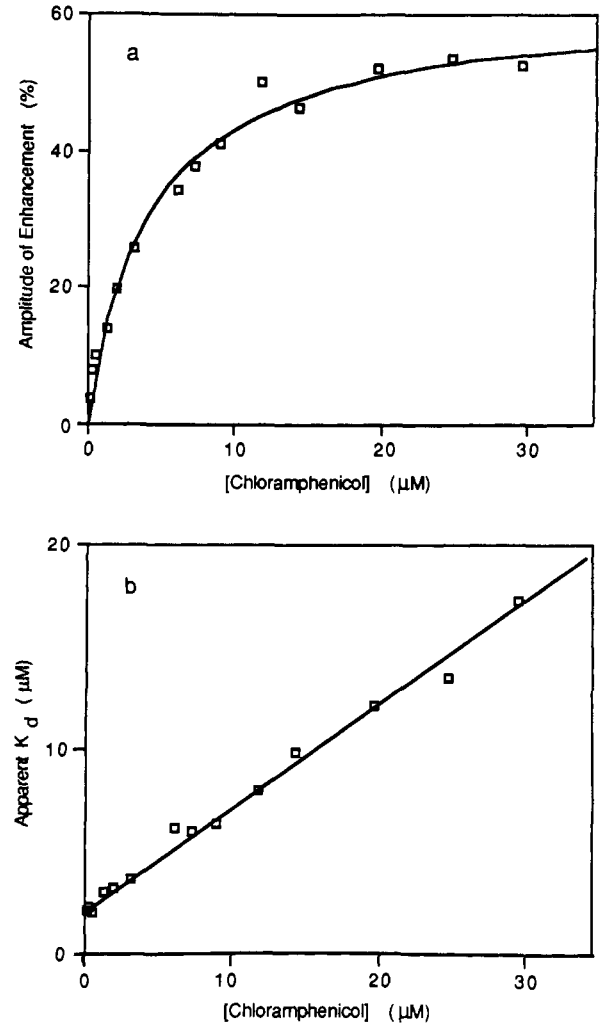


FIGURE 3: Secondary plots of the competition binding assay data for chloramphenicol and *p*-cyano-CM. (a) Each point represents the fluorescence enhancement observed in the CAT_{III} sample on titration with *p*-cyano-CM. Each amplitude change corresponds to the degree of saturation of the protein at a given concentration of chloramphenicol, such that the plot describes a simple binding curve for chloramphenicol. The accumulation of data and curve-fitting procedures are described under Experimental Procedures. (b) Each data point is the apparent K_d found from the nonlinear regression fits to the primary titration curves.

reaction, such that submicromolar concentrations of CAT_{III} monomer are required. The consequent loss of fluorescence signal, together with the necessity to use chloramphenicol concentrations near K_d (4 μM) to restrict association rates to a measurable range ($<\sim 600\ s^{-1}$), requires that the stopped-flow instrument operate close to its limits of sensitivity. Hence, the calculation of values for k_1 and k_{-1} ($4.8 \times 10^7\ M^{-1}\ s^{-1}$ and 198 s^{-1}, respectively) was complemented by an independent measure of k_{-1} (207 s^{-1}) by displacement of chloramphenicol from the binary complex with CAT_{III} by *p*-cyano-CM (Figure 4a). As $K_d = k_{-1}/k_1$, these rate constants reflect a dissociation constant of 4.2 μM, in agreement with the dissociation constants measured by fluorometric titration (4.3 μM) and derived from steady-state kinetic analysis (4.0 μM), all of which are summarized in Table I.

A reevaluation of K_d for chloramphenicol and CAT_{III} by equilibrium dialysis produced data which, when initially plotted according to Scatchard (1949), produced a value of 10.7 μM and a stoichiometry of 0.94 by linear regression analysis in quadruplicate experiments. However, inspection of the data revealed a slight, but reproducible, deviation from linearity,

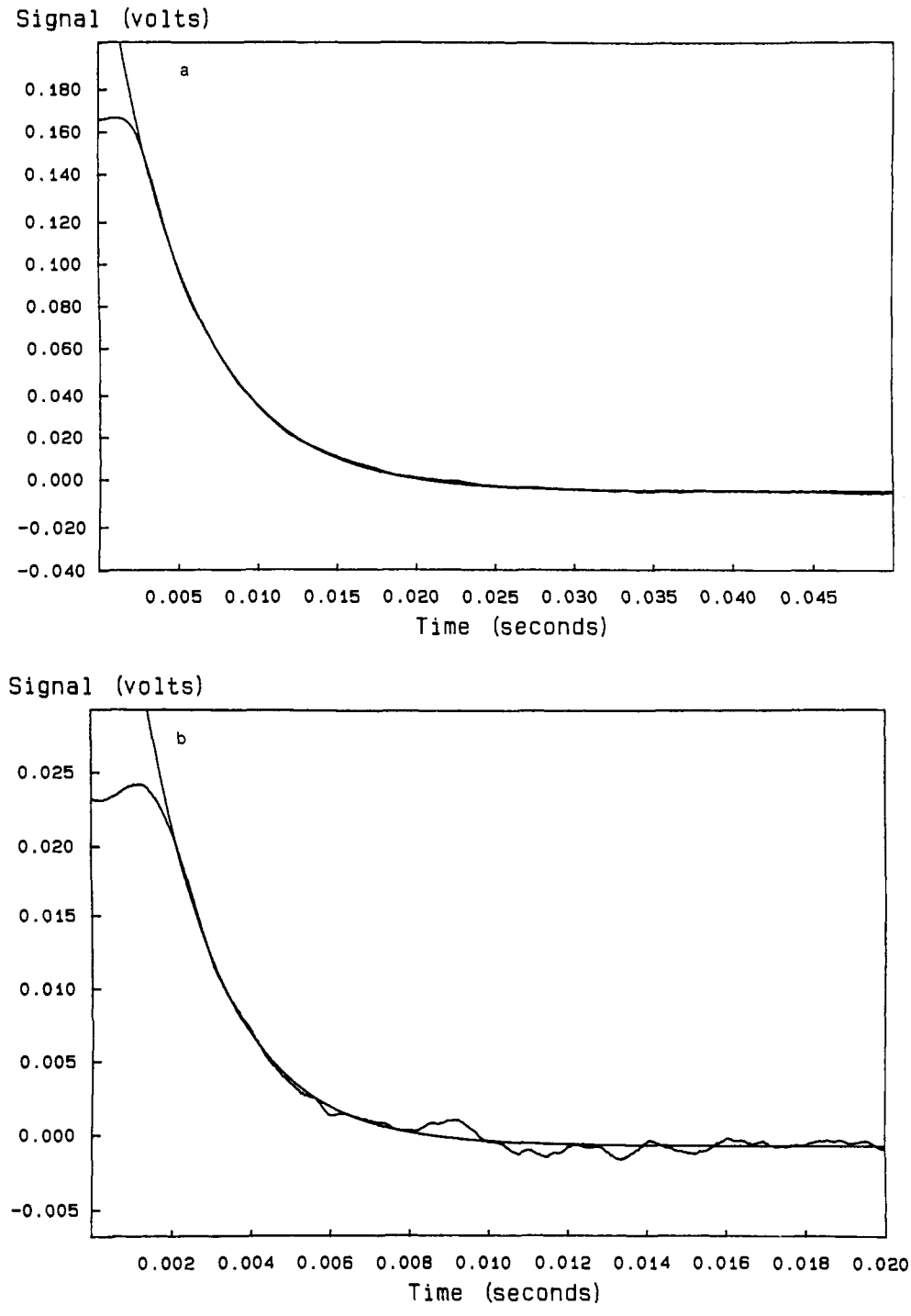


FIGURE 4: Stopped-flow records of the dissociation of chloramphenicol from binary and ternary complexes. (a) Dissociation of chloramphenicol from the binary complex with protein alone. One syringe contained 100 μM *p*-cyano-CM, and the other contained 50 μM chloramphenicol and 8 μM CAT_{III}. An exponential fit is superimposed on the decay phase and yields a rate constant of 207 s^{-1}. The data shows a 17% enhancement of intrinsic protein fluorescence. (b) Dissociation of chloramphenicol from the ternary complex with protein and the nonhydrolyzable ethyl-CoA. One syringe contained 400 μM ethyl-CoA and 100 μM *p*-cyano-CM and the other contained 400 μM ethyl-CoA, 50 μM chloramphenicol, and 8 μM CAT_{III}. The superimposed exponential fit yields a rate constant of 608 s^{-1}. The data shows a 2.5% enhancement of fluorescence.

which demanded further attention in the light of the dangers inherent in the uncritical interpretation of nonlinear Scatchard plots (Edsell & Wyman, 1958; Steinhardt & Reynolds, 1969; McGhee & von Hippel, 1974; Zierler, 1989). To avoid such misinterpretation, the data sets obtained were also plotted as a simple binding curve and fitted by nonlinear regression analysis (Figure 5). Although fitting the data to a one-term hyperbola yielded a dissociation constant of 11.5 μM, comparable to that calculated from the Scatchard plot, the calculated curve does not fully represent all of the data points. A much improved correspondence between the experimental data and the theoretical curve was found upon use of a two-term hyperbolic expression. The latter produced dissociation constants of 3.6 μM and 74 μM, the former comparing favorably with that obtained by use of the alternative techniques (Table I), though the failure of the amplitude term to correlate to a stoichiometry of 2 (the number of binding sites classes per monomer) remains an anomaly for the equilibrium dialysis experiment. An explanation may lie with the progressively larger standard errors of the primary data as the concentration of chloramphenicol is increased. The one-site hyperbola is biased by data at low concentrations whereas the two-site

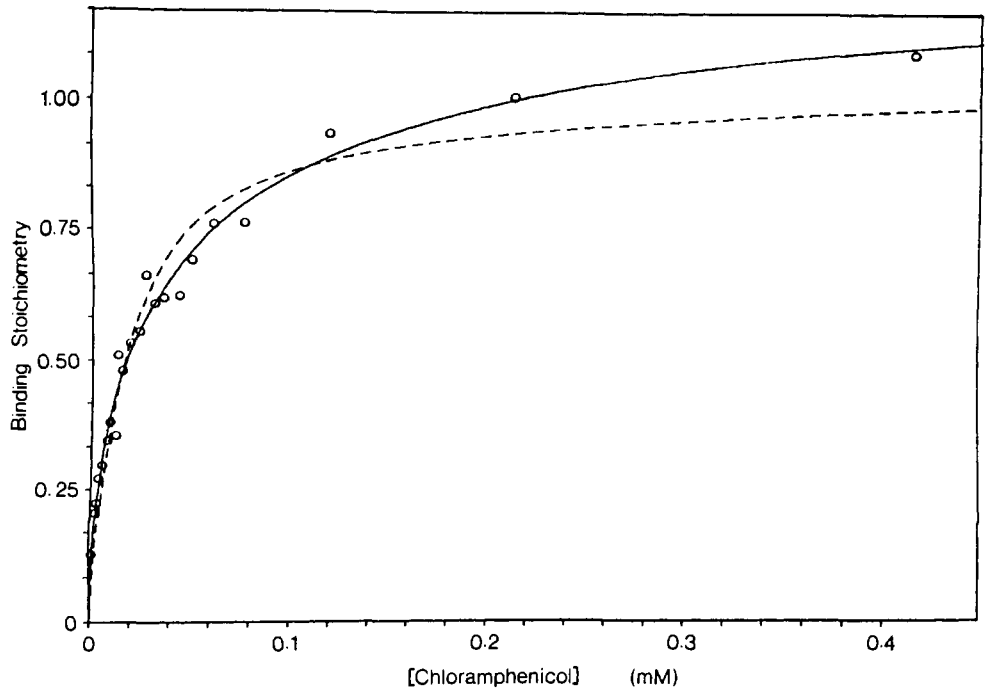


FIGURE 5: Analysis of chloramphenicol binding data obtained by equilibrium dialysis. The scale of the ordinate gives the molar ratio of chloramphenicol bound to CAT monomers (12 μM). Analysis of the data by nonlinear regression to a one-term hyperbola (dashed line) gives a binding constant of 11.5 μM, but the theoretical curve does not fully represent all data points. Fitting a two-term hyperbolic function to the same data (solid line) yields a closer agreement between theoretical curve and experimental data, with chloramphenicol binding constants of 3.6 μM and 74 μM. Analysis of the deviation of fitted curves for each model from the experimental data revealed a random pattern of residuals for the two-site assumption as compared with a systematic variation about the approximation for single-site binding (data not shown).

assumption gives equal weight to the smaller number of data points with inherently higher errors. Collateral evidence for the binding of chloramphenicol at two sites comes from crystallographic data on *p*-iodo-CM, an analogue of chloramphenicol with the *p*-nitro group replaced by iodine, which binds at a second site, one which accommodates the adenine moiety of CoA in the refined structure of the CAT·CoA binary complex, (Leslie et al., 1988; Leslie, 1990).

Binding of Acetyl-CoA in the Binary Complex with CAT_{III}. The direct measurement of the dissociation constant for the CAT_{III} acetyl-CoA binary complex has been hampered by the thioesterase activity of CAT_{III} and the time required for equilibrium dialysis. The finding that the binding of acetyl-CoA to CAT_{III} is accompanied by a 24% enhancement of intrinsic protein fluorescence (Ellis et al., 1991) allowed the direct measurement of the dissociation constant by a fluorometric titration (Figure 6). The value obtained by this technique (28 μM) is in close agreement with the derived value (31 μM) obtained from the steady-state kinetic analysis of the forward transacetylation reaction (Table I). At 25 °C, the association and dissociation rate constants for acetyl-CoA binding to CAT_{III} were too fast to measure by stopped-flow techniques.

Binding of Acetyl-CoA in Nonproductive Ternary Complexes. The apparent inequality of binary and ternary complex dissociation constants (K_d and K_m) for acetyl-CoA was examined by fluorometric titration. Chloramphenicol analogues substituted at the primary (C-3) hydroxyl position (the acyl acceptor in the transacetylation reaction) were chosen to identify the origin of the apparent substrate synergism in the system, given that the structure of CAT_{III} in both binary complexes appears to be identical. Accordingly, the 3-hydroxyl was replaced by hydrogen (3-deoxy-CM) and iodine (3-iodo-CM) and substituted by acetyl (3-acetyl-CM), the latter being a product of the forward transacetylation reaction (Figure 2). In each case, the K_d for the inhibitor in the binary

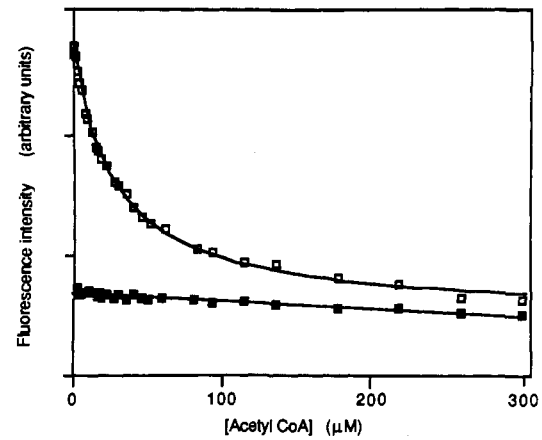


FIGURE 6: A typical binding curve from the automatic titration of CAT_{III} with acetyl-CoA. The titration was carried out as described under Experimental Procedures. The data (open squares) represent a total of 24% enhancement of intrinsic protein fluorescence. The superimposed fit to a hyperbolic expression yields a K_d for acetyl-CoA of 28.1 ± 1.8 μM. The transmission (filled squares) was monitored simultaneously with the fluorescence throughout the titration.

complex with CAT_{III} was determined and a concentration of 3–6 times that value was included with the CAT_{III} in titrations for the measurement of the dissociation constant (equivalent to K_m) of acetyl-CoA from the ternary complex.

Table II shows the binding data for acetyl-CoA in the various nonproductive ternary complexes. 3-Deoxy-CM is the only inhibitor of the three which does not affect the binding of acetyl-CoA. Thus, deletion of the primary hydroxyl, with the consequent loss of its hydrogen bond made to the $N^{\epsilon 2}$ of the catalytic residue, His-195, removes the indirect interaction between substrates responsible for the 3-fold decrease in affinity for acetyl-CoA in the ternary complex. Although substitution of the 3-hydroxyl of chloramphenicol by the much larger acetyl group produces a ternary complex in which the

K_m for acetyl CoA is 102 μM, the effect may be due either to (a) adoption of an alternate binding mode, comparable to that found in the productive complex with chloramphenicol, or (b) steric hindrance resulting from the close proximity of two acetyl groups.

Since the replacement of the primary (C-3) hydroxyl of chloramphenicol with iodine (3-iodo-CM) is less extreme than either removing it (3-deoxy-CM) or replacing it with a large acetyl group, it was anticipated that the iodo substitution might reproduce the effects of chloramphenicol binding save for the absence of a hydrogen bond to the imidazole of His-195 (Cullis et al., 1991). The observed dissociation constant of acetyl-CoA from the resultant ternary complex (61 μM) is consistent with this reasoning, the iodo group being accommodated into the active site but without producing the complete interaction between substrates necessary to induce a 3-fold difference.

Binding of Chloramphenicol in Nonproductive Ternary Complexes. The measurement of the association and dissociation rate constants for chloramphenicol in two nonproductive ternary complexes allowed the calculation of the equilibrium dissociation constant (denoted as K_m, for consistency with steady-state kinetic parameters) of chloramphenicol from that complex. The nonproductive ternary complexes utilized either CoA, the product of the forward reaction, or the thioether analogue of acetyl-CoA (ethyl-CoA) as the second ligand. These were present in both drive syringes to avoid artifacts due to dilution. For the measurement of dissociation rate constants, chloramphenicol was displaced from the preformed ternary complex by *p*-cyano-CM (Figure 4b).

Table III summarizes the results obtained. It is clear that it is the dissociation rate constant (k_{-1}) which changes between different complexes and not the association rate constant. CoA changes neither the binding affinity of CAT_{III} for chloramphenicol nor the kinetics of the process. Ethyl-CoA, which itself has binding characteristics comparable to acetyl-CoA and results in a similar enhancement of protein fluorescence (data not shown), increases the rate of dissociation of chloramphenicol from the ternary complex to ~600 s^{-1}. This accounts for the 3-fold decrease in association constant ($1/K_m$ as compared with $1/K_d$) observed in the steady-state measurements.

The fact that the binding of the CoA does not have an effect similar to that of its ethylated and acetylated derivatives may find a parallel in the observation that the refined structures of the binary complex of CAT_{III} with either CoA or chloramphenicol are essentially identical. It follows that only certain ternary complexes used in this study, those consisting of CAT_{III} and chloramphenicol plus either the authentic acyl donor or a structural mimic, should produce the effects of negative cooperativity.

Discussion

In summary, the directly measured dissociation constants for the binary complexes for CAT_{III} and its substrates compare well with the corresponding values derived from the steady-state kinetic analysis of the system. The agreement between the estimates of the parameters based on the rapid equilibrium, random order model, and the measured equilibrium constants may be taken as further evidence for the postulated kinetic mechanism.

From a practical point of view an additional binding assay for ligands at the active site of CAT_{III} has many applications, particularly with catalytically deficient mutant proteins. Fluorescence-based methods also eliminate the need for equilibrium dialysis which is wasteful of protein and may only be used for enzymes and substrates which are stable for long periods of time under standard conditions of temperature and pH.

A more important conclusion from these studies concerns the detection of a state of enzyme and substrates en route to the formation of the transition state. It is accepted that it is most catalytically advantageous for the active site of an enzyme to be complementary to the transition state of the reactants, rather than any other bound species, as the activation energy for the catalytic step is lowered, allowing the reaction to proceed with consequent rate acceleration [discussed by Burbaum et al. (1989)]. In the case of CAT_{III}, a modeled tetrahedral intermediate [Leslie and Moody, unpublished results; Lewendon et al., 1990], based on the chemical mechanism and structures of the binary complexes, produces very small changes in protein side-chain conformations (maximum movement ~0.2 Å) but a larger movement (0.7 Å) in the 3-hydroxyl oxygen of chloramphenicol, relative to its position in the binary complex. Also, additional interactions become available in the tetrahedral intermediate as the methyl group attached to the tetrahedral carbon is in van der Waals contact with the side chains of Phe-33 and Phe-158, and the oxygen atom of the tetrahedral carbon participates in a hydrogen-bond network involving the hydroxyls of Ser-148 and Thr-174, the secondary hydroxyl of chloramphenicol, and an ordered water molecule (Leslie, 1990; Lewendon et al., 1990).

The observation that two different ligands may bind to a protein with mutual effects on their respective association constants is by no means novel, resulting in what has been described as "free energy coupling" (Weber, 1975). The degree of cooperativity (K_c) is given by the ratio of the binding constants for each ligand in the ternary and binary complexes. The corresponding free energy change is ΔG_c° such that negative cooperativity gives $\Delta G_c^\circ > 0$. Although much attention has been given to circumstances wherein substrates bind more lightly (positive cooperativity), rather less is known in detail about systems analogous to CAT which display negative cooperativity. A case in point is that of dihydrofolate reductase wherein the negative cooperativity between folinic acid and coenzyme (Birdsall et al., 1981) can now be understood more readily in the light of a detailed kinetic and thermodynamic analysis of the system (Benkovic et al., 1988).

Returning to CAT, chloramphenicol, and acetyl-CoA, it seems likely that a prerequisite to the formation of the ternary complex, on binding the second substrate, is a deviation of ligand conformations (and/or those of the enzyme) from conformations which exist in each of the binary complexes, the phenomenon of "strain". The reciprocal 3-fold decreases in affinities of enzyme for each substrate in the ternary complexes corresponds to a free energy change of +0.65 kcal·mol^{-1} for the system as a whole. It follows that the energetically less favorable conformations in the ternary complex may proffer new interactions of CAT with acyl donor and acceptor and thus facilitate formation of the transition-state intermediate, which in turn is stabilized by optimal complementarity to the enzyme. The possible physical factors involved in such negative substrate cooperativity cannot be defined without a reliable structure of a ternary complex equivalent to that which obtains on the actual reaction pathway to the tetrahedral intermediate. Since the reaction catalyzed by CAT is reversible (although energetically unfavorable in the direction of thioester formation), it is possible that an increased rate of dissociation for chloramphenicol from the ternary complex serves as a mechanism to speed loss of product, particularly so since the rate of loss of chloramphenicol from the binary complex (~200 s^{-1}) is comparable to the overall turnover

($\sim$100 s^{-1}) in the reverse direction (data not shown). There is also scope for studying the properties and structure of the reverse reaction ternary complex of CAT, CoA, and 3-acetylchloramphenicol (Figure 1). Such information and the determination of all of the microscopic rate constants by stopped-flow fluorometry should allow a more comprehensive view of the conclusions arising from the present study.

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Identification of an Inhibitor of Microtubule Assembly Present in Juvenile Brain Which Displays a Novel Mechanism of Action Involving Suppression of Self-Nucleation†

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ABSTRACT: An inhibitor of microtubule assembly has been identified and partially purified from microtubule-depleted brain extracts from day-old chicks and 4-month-old calf. This inhibitor suppresses the self-nucleation of microtubules in vitro with minimal effect upon the final extent of assembly. It may have a developmental role in vivo as it is not detected in adult brain from either cattle or rabbit.

Cytoplasmic microtubules can be highly dynamic, with half-times of approximately 10 min (Schulze & Kirschner, 1987), and are not randomly distributed within the cell. They are generally nucleated by and radiate from specialized structures known as microtubule organizing centers (MTOCs; Brinkley et al., 1981; Karsenti et al., 1984). Assembly kinetics predict a free steady-state concentration of unpolymerized tubulin, yet various tissues contain a substantial concentration of free tubulin (Pipeleers et al., 1977) which exceeds the values observed in vitro.

Microtubule assembly may be regulated at a number of different levels. Endogenous inhibitors have been reported

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