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Substrate and Product Inhibition Initial Rate Kinetics of Histone Acetyltransferase†

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ABSTRACT: Initial velocity and product inhibition kinetics of the histone acetyltransferase (EC 2.3.1.48) reaction indicate that the rat liver nuclear enzyme operates under a rapid equilibrium ordered bireactant mechanism. Histone adds first to the enzyme, and under the conditions of the experiment $K_a = 0$ as acetyl coenzyme A (CoA) concentration approaches saturating conditions. The K_m for acetyl-CoA was 2.10 ± 0.48 μM. Inhibition with acetyllysine resulted in a K_{iq} for the enzyme·acetyllysine complex of 1.96 ± 0.30 mM. Inhibition with CoA yielded K_{ip} for the ternary complex of 3.19 ± 0.48 μM. These results indicate that the enzyme activity is comparatively independent of histone concentration, and, since the enzyme is sensitive only to acetyl-CoA and CoA concentrations, the enzyme will tend to maintain histones in the acetylated state.

In chromatin, histones are organized in a repeating structure consisting of approximately 200 base pairs of DNA associated with one molecule of histone H1 and two molecules each of histones H2a, H2b, H3, and H4 (Kornberg & Thomas, 1974; Thomas & Kornberg, 1975; Simpson & Bustin, 1976; Weintraub et al., 1975; Shaw et al., 1976; Simpson & Whitlock, 1976). Since a distinctive feature of histones is their large complement of basic residues, particularly in the N-terminal regions, it is generally accepted that the histones interact with DNA through ionic interactions. Histones may undergo a series of postsynthetic modifications, including acetylation and methylation of lysyl residues and phosphorylation of seryl residues. The modifications of lysyl residues in particular can lead to altered positive charge densities in the critical regions

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which interact with the DNA and a generalized weakening of the DNA–histone interaction. In support of this, Wallace et al. (1977) have demonstrated that acetylation of chromatin with acetic anhydride leads to a decrease of DNA melting temperature with increasing degree of acetylation, presumably due to a decrease in the stability of the nucleosome. Indeed, since the discovery of histone acetylation by Allfrey et al. (1964), a great deal of evidence has accumulated which correlates histone acetylation with an increase in DNA-dependent RNA synthesis. Marushige (1976) has demonstrated that chemical acetylation of chromatin leads to an increase in its availability as a template for RNA synthesis. Others (Levy-Wilson et al., 1977; Davie & Candido, 1978) have applied the technique of Gottesfeld et al. (1974) in which chromatin digested with DNase II, coupled with Mg^{2+} precipitation, leads to a fraction of chromatin enriched in actively transcribing sequences and have found a preferential association of acetylated histones with this chromatin fraction. In addition, hyperacetylation of histones, achieved by inhibition of histone deacetylase with sodium butyrate, has been shown to induce, in nontranscribable chromatin, the pattern of susceptibility to DNase I digestion normal for chromatin engaged in transcription (Simpson, 1978; Vidali et al., 1978).

In contrast to the large amount of experimental evidence linking acetylation with gene transcription, very little work has been performed on the enzymatic machinery responsible for histone acetylation. Histone acetyltransferases (EC 2.3.1.48) have been purified from various tissues to varying degrees of purity (Gallwitz, 1971; Lue et al., 1973; Candido, 1975; Cano & Pestaña, 1979), but an investigation of the process governing the regulation of this activity has generally been lacking. Recently, however, inhibitors of both acetyltransferase (obtained from A. Salina) (Cano & Pestaña, 1976) and deacetylase (Reeves & Candido, 1979) have been described. In our lab, recently, we have been interested in the regulation of acetyltransferase activity, and as an initial step in this investigation, we describe the kinetic interaction of histone acetyltransferase, its substrates, acetyl-CoA and histone, and the products of the reaction, CoA and N^{ϵ}-acetyllysine.

Materials and Methods

Tritiated acetyl-CoA (14 Ci/mmol) was obtained from ICN (Irvine, CA) and diluted with an appropriate amount of cold acetyl-CoA, obtained from Sigma (St. Louis, MO), to give a specific activity of 2.5 Ci/mmol. P-81 phosphocellulose filter paper disks (2.4 cm) were purchased from Whatman (Clinton, NJ). Calf thymus histones (Sigma) were used as is. CoA was obtained from Sigma. N^{ϵ}-Acetyllysine was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of reagent grade.

Purification of Histone Acetyltransferase. The details of the purification of rat liver histone acetyltransferase are given elsewhere (J. E. Wiktorowicz, K. L. Campos, and J. Bonner, unpublished results). The enzyme used in these experiments was purified at least 50-fold and free of histone.

Initial Rate Assay of Histone Acetyltransferase. The initial rates of enzyme activity were determined by a modification of the procedure of Horiuchi & Fujimoto (1975). In a final volume of 60 μL were contained (final concentration) histone (variable concentration), [^{3}H]acetyl-CoA (2.5 Ci/mmol) (variable), 20 mM Tris-HCl (pH 7.9), 40 mM KCl, 40 mM NH_4Cl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, distilled deionized water, and 10 μL of enzyme. The components of the reaction mixture minus acetyl-CoA were preincubated at 37 °C for 3 min, as was the stock acetyl-CoA. Where product inhibitions were performed, products were substituted for water, with one substrate varied about its K_m and the other substrate fixed at or near its K_m. The reaction was started by the addition of the appropriate amount of acetyl-CoA and allowed to proceed for 3 min at 37 °C. The pH of the final reaction mixture at 37 °C was 7.39 ± 0.03. Cold (nonisotopic) acetyl-CoA (~25-fold excess) and histone (to bring the final histone concentration to ~1.5 mg/mL) were added to dilute the isotope incorporation, and the samples were immediately immersed in ice. An aliquot (57 μL) of the final reaction mixture (68.5 μL) was layered onto phosphocellulose disks and immersed in 50 mM sodium carbonate buffer (pH 9.2) at 37 °C for 30 min. A final wash in buffer at room temperature for 5 min was followed by drying with ethanol and ether. The filter papers were counted in Aquasol (Beckman). The efficiency of counting in this system was routinely 21%. Appropriate enzyme blanks were performed, and one unit of enzyme activity is defined as that amount of enzyme which will utilize 1 pmol of acetyl-CoA per minute at 37 °C and pH 7.4. Each kinetic experiment was repeated at least 4 times, and the data from each were processed independently.

Analysis of Initial Rates and Computation of Kinetic Constants. Data analysis was performed on a Digital PDP 11/34 by using a modification of programs written by Murphy et al. (1979), and kinetic analyses were performed by using a modified version of programs written by Cleland (1979).

Reciprocal initial velocities from substrate kinetic experiments were plotted vs. reciprocal substrate concentrations, and the data were fitted to a sequential initial velocity pattern (eq 1) as well as to a rapid equilibrium ordered mechanism (eq 2). The data from the product inhibition studies were fitted to expectations of competitive inhibition (eq 3), and uncompetitive inhibition (eq 4).

$$v = \frac{VAB}{K_{ia}K_b + K_bA + K_aB + AB} \quad (1)$$

$$v = \frac{VAB}{K_{ia}K_b + K_bA + AB} \quad (2)$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \quad (3)$$

$$v = \frac{VA}{K + A(1 + I/K_{ii})} \quad (4)$$

Nomenclature is that of Cleland (1963). All equations were fitted by assuming equal variance for the velocities.

Results

Figure 1 demonstrates the time course dependence of the acetylation reaction at various combinations of acetyl-CoA and histone concentrations, ranging from the lowest to the highest concentration combinations used in the following kinetic determinations. It is clearly demonstrated that the reaction is first order for at least 4 min, and significant departure from first order occurs after about 5 min with the lowest concentration combination of substrates used. Accordingly, all reactions were terminated after 3 min of reaction time (see Materials and Methods). The greatest utilization of substrate occurs at the highest combination of substrate concentrations (8.00 μM Ac-CoA, 0.83 mg/mL histone) and represents less than 5% utilization of substrates.

The data obtained from these experiments were fitted separately to eq 1 and 2. The programs generated by Cleland (1979) calculate the parameters K_a, K_b, K_{ia}, and K_{ib} and their standard errors as well as the standard deviation (σ) of the fit. The parameters, as fit to eq 1 and 2, the standard errors,

Table I: Comparison of Computed Parameters between Intersecting Sequential and Rapid Equilibrium Ordered Mechanisms[a]

kinetic mechanism	K_a (mg/mL)	CV[b]	K_{ia} (mg/mL)	CV	K_b (μM)	CV	σ
intersecting sequential	0.010 ± 0.008	0.823	0.147 ± 0.165	1.12	3.48 ± 1.81	0.520	6.08
rapid equilibrium ordered			0.222 ± 0.069	0.311	2.10 ± 0.48	0.227	6.25

[a] By convention, in an ordered mechanism, A is defined as the first substrate to bind, B is the second substrate to bind, Q (acetylated histone) is the product remnant of A, and P is the product remnant of B. A = histone; B = acetyl-CoA. Values are weighted averages from three separate kinetic experiments. [b] CV = coefficient of variation = SE ÷ parameter value.

Table II: Inhibition Constants[a]

inhibitor	variable substrate	fixed substrate	K_{is}	K_{ii}	inhibition[b]
N^{ϵ}-acetyllysine	acetyl-CoA	histone	2.50 ± 0.55 mM		C
N^{ϵ}-acetyllysine	histone	acetyl-CoA	1.96 ± 0.18 mM		C
CoA	acetyl-CoA	histone	2.77 ± 0.49 μM		C
CoA	histone	acetyl-CoA		5.43 ± 0.86 μM	UC

[a] Constants were weighted averaged obtained from at least three separate experiments for each inhibitor–variable substrate combination. [b] C = competitive inhibition fit to eq 3. UC = uncompetitive inhibition fit to eq 4.

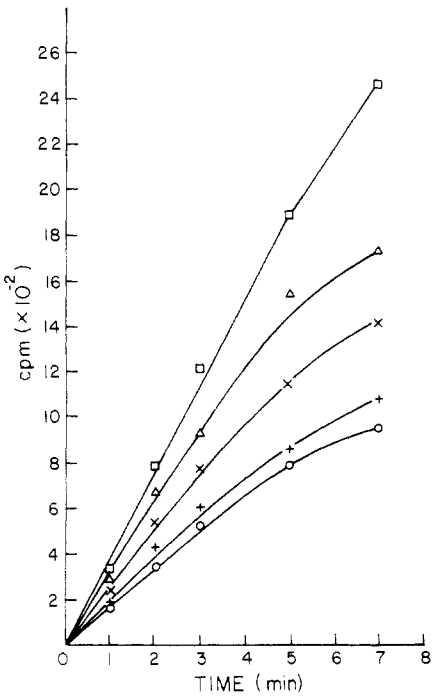

FIGURE 1: Time course dependence of acetylation. The assays are performed as indicated under Materials and Methods. After appropriate times 50 μL out of an initial volume of 300 μL of reaction mix is removed, spotted onto phosphocellulose filter papers, and processed as usual. Concentration combinations of acetyl-CoA (μM) and histone (mg/mL) are respectively in increasing velocity rates: (○) 1.60, 0.36; (+) 2.00, 0.42; (×) 2.67, 0.50; (△) 4.00, 0.63; (□) 8.00, 0.83.

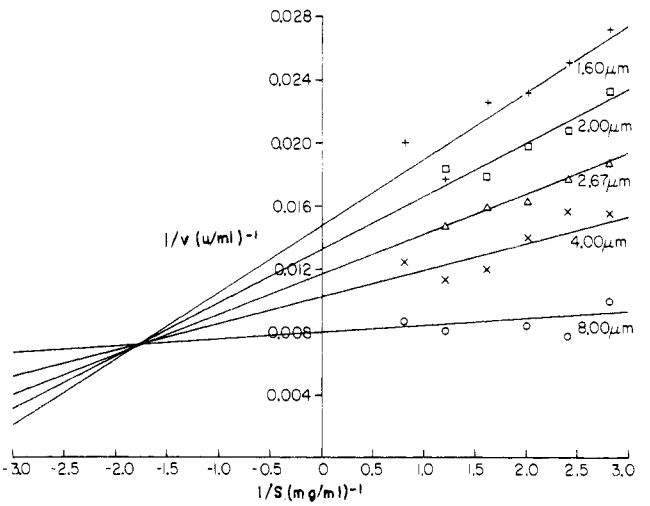

FIGURE 2: Reciprocal plot of velocity vs. histone. See Materials and Methods for details. The graph demonstrates the intersecting (sequential) nature of the kinetic mechanism. Each line represents a different concentration of acetyl-CoA as indicated in the figure.

and σ are presented in Table I. It is clear from this table that K_a is very small compared to K_b, and indeed, when the data are fit to the generalized eq 1 for a sequential mechanism, K_a is very small compared to K_b. In addition, the standard errors of K_a were routinely greater than K_a, indicating complete lack of significance for K_a. This implies that the K_aB term from eq 1 may be ignored, and the resultant eq 2 describes a rapid equilibrium ordered mechanism in which $K_a = 0$ when B approaches saturating conditions. As can be seen from Table I, no significant change in σ occurs when the data are fitted to eq 2. In fact, the computed parameters are improved, as evidenced by the lower coefficients of variation (CV). The fit to a rapid equilibrium ordered mechanism from one data set, typical of all of the data sets, is presented in graphic form in Figure 2. The initial rates are clearly intesecting, justifying the use of eq 1 and 2.

Product inhibition was examined by using coenzyme A (CoA) and N^{ϵ}-acetyllysine as the products of the reaction. N^{ϵ}-Acetyllysine was used to avoid the difficulties of obtaining fully N-acetylated histones in quantities great enough for the experiments. The initial velocity patterns obtained from the product inhibition experiments are presented in Figure 3. This figure demonstrates that when either substrate is varied, acetyllysine behaves as a competitive inhibitor. On the other hand, CoA behaves as a competitive inhibitor when acetyl-CoA is varied, but as an uncompetitive inhibitor when histone is varied.

The inhibition constants (K_{ii} and K_{is}) for CoA acetyllysine are presented in Table II. Slope replots to determine K_{iq} for acetyllysine with variable histone resulted in K_{iq} = 1.96 ± 0.30 mM. Intercept replots to determine K_{ip} from CoA inhibition with variable histone resulted in K_{ip} = 3.21 ± 0.24 μM.

Discussion

Purified histone acetyltransferase from rat liver nuclei operates according to an ordered bireactant mechanism, with histone adding before acetyl-CoA. If the data were fitted to a rapid equilibrium ordered mechanism assuming acetyl-CoA to be the first substrate to add, in all data sets determined K_b (histone) and K_{ia} (for the enzyme-acetyl-CoA complex) are negative values (data not shown). Both σ and the CV's of the constants are significantly greater than if the assumption of

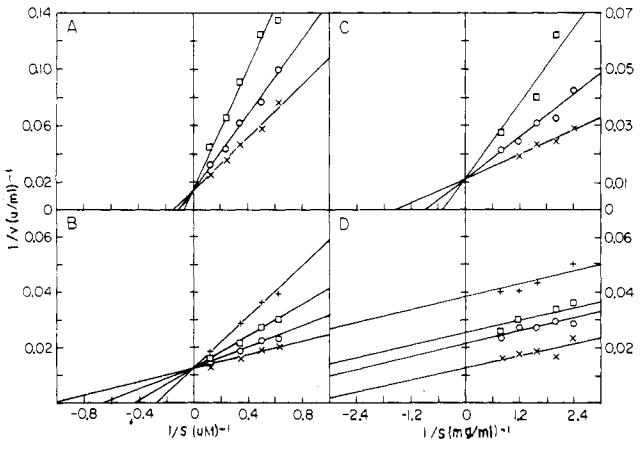

FIGURE 3: Product inhibition kinetics. Each reciprocal plot represents a series of experiments (performed at the same time) in which one substrate concentration was fixed with variable inhibitor while the other substrate was varied. (A) Acetyllysine inhibition [(×) 0 mM; (○) 1.98 mM; (□) 3.19 mM] with variable acetyl-CoA and fixed histone (0.7 mg/mL). (B) CoA inhibition [(×) 0 μM, (○) 2.5 μM, (□) 5.0 μM, (+) 10.0 μM] with substrate combinations as in (A). (C) Acetyllysine inhibition [(×) 0 mM, (○) 1.43 mM, (□) 3.19 mM] with variable histone and fixed acetyl-CoA (2.5 μM). (D) CoA inhibition [(×) 0 μM, (○) 2.5 μM, (□) 5.0 μM, (+) 10.0 μM) with substrate combinations as in (C). Acetyl-CoA concentration was 1.25 μM.

a histone binding prerequisite for activity is made. This implies that the binding of histone induces a conformational change in the active site which then allows acetyl-CoA to bind. This is supported by the CoA inhibition pattern with histone as the varied substrate (Figure 3D) in which only an intercept effect is seen. Such a pattern will only be observed when the inhibitor binds after the varied substrate, i.e., to the EA complex. Under the conditions of the experiment, therefore, CoA acts as a dead-end inhibitor. The equation which describes the presence of an EAP dead-end inhibitor under rapid equilibrium assumptions is given by eq 5.

$$v = \frac{VAB}{K_{ia}K_b(1 + p/K_{ip}) + K_bA + AB} \quad (5)$$

Since acetyllysine inhibits either substrate in a competitive manner (Figure 3A,C), no dead-end complex between enzyme, acetyllysine, and acetyl-CoA occurs (EBQ), as this would lead to an inhibition with slope and intercept effects (mixed type). The competitive inhibition implies that the acetyl groups of acetyl-CoA and acetyllysine are mutually exclusive, i.e., both are competing for the only acetyl binding site in the active center. This also indicates that acetyllysine binding is not sufficient to induce the conformational change prerequisite for acetyl-CoA binding.

The physiological significance of the kinetic mechanism depends in part on the specificity of the enzyme for the histone substrate. Histones may exist in the nucleus as free protein, in a "histone pool", or in the nucleosome form. From the proposed ordered mechanism and the K_m and K_{ip} determined from the experiment, it follows that the enzyme activity will be controlled by the concentration of acetyl-CoA and CoA in the nucleus (since it is apparently insensitive to the concentration of histone, i.e., $K_a = 0$) and that free histone will probably tend to exist in the acetylated state. This has in fact been observed by investigators who have shown that the histone in the nuclear "pool" is immediately acetylated (<1 min) upon entry into the nucleus and achieves a steady-state after 30 min due to the action of histone deacetylase (Jackson et al., 1976; Woodland, 1979). The action of the purified enzyme on histones in the nucleosome, however, is not clear and is currently under investigation.

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