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Kinetic Mechanism of the Reaction Catalyzed by Nuclear Histone Acetyltransferase from Calf Thymus†

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ABSTRACT: The kinetic mechanism for calf thymus histone acetyltransferase A has been determined from the initial velocity studies. The kinetic patterns at low substrate concentrations suggest that the reaction proceeds via two half-reactions as in a ping-pong pathway with the formation of an acetyl-enzyme intermediate. Such acetyl-enzyme has been isolated and found to be chemically competent. In addition, product inhibition patterns by coenzyme A are consistent with a hybrid ping-pong mechanism. These findings indicate that the acetyltransferase A from calf thymus has two separate and independent binding sites, one for each of the two substrates. Consequently, the mechanism constructed for the acetyltransferase A catalyzed reaction may be described as a double-displacement, two-site ping-pong mechanism.

Nuclear histone (acetyltransferase A) (EC 2.3.1.48) catalyzes the transfer of acetyl group from acetyl-CoA[1] to histones according to the following equation:

$$\text{acetyl-CoA} + \text{histone} \rightleftharpoons \text{acetylhistone} + \text{CoA}$$

The acetylation takes place at the ϵ-amino groups of lysines located within the first 25 amino acid residues of the N-termini of the histones (DeLange & Smith, 1975; Allfrey, 1977). As a result of the modification, the positive charge density of the histones is drastically reduced and thus their ionic interactions with DNA. Such change of interaction between histones and DNA is important in the control of structure and function of chromatin, which in the eukaryotic cell is composed of repeating units of nucleosomes, each of which consists of two each of the four histones H2a, H2b, H3, and H4, with 146 base pairs of DNA wrapped around the octameric histone core particle. The nucleosomes are linked together by 40–60 base pairs of DNA with the association of one molecule of histone H1 (Kornberg, 1977; Felsenfeld, 1978). The amino acid sequences of the four nucleosomal histones have been highly conserved during evolution (DeLange & Smith, 1975; Isenberg, 1979). However, extensive postsynthetic chemical modifications such as acetylation, phosphorylation, methylation, and ADP-ribosylation have been noted (Allfrey, 1977). The degree and extent of modification on the histones would certainly change the ionic interactions between the histones and DNA in the nucleosomes, thus providing a means of control of chromatin structure and function (Allfrey, 1977; Alberts et al., 1977; Dixon et al., 1975). In particular, acetylation has been speculated to correlate with increased transcriptional activity of genetic codon by RNA polymerase (Allfrey, 1977; Chahal et al., 1980). Such speculation has been supported by the observations that histones associated with template-active chromatin are preferentially acetylated (Wong & Alberts, 1977; Levy-Wilson et al., 1977; Davie & Candido, 1978; Simpson, 1978; Nelson et al., 1978; Vidali et al., 1978).

The enzyme system involved in the important acetylation process has not been fully investigated in detail because of its minute amount in the cell and its unstability on purification. Recently, nuclear acetyltransferase has been purified to a high degree from calf thymus (Belikoff et al., 1980) and rat liver (Wiktorowicz & Bonner, 1982), and some general properties have been investigated. We present here detailed kinetic studies on the reaction mechanism in an attempt to understand the regulation and involvement of the enzyme in gene expression (Wong, 1980). This report will show that the reaction follows a double-displacement pathway.

Materials and Methods

Materials. Calf thymus histones (a mixture of H1, H2a, H2b, H3, and H4), CoA, acetyl-CoA, N^{ϵ}-acetyllysine, and bovine serum albumin were purchased from Sigma Chemical Co. Sea urchin sperm histones were prepared as described previously (Wong, 1980). P-81 phosphocellulose paper was obtained from Whatman while [^{3}H]acetyl-CoA (10 Ci/mmol) was from ICN. All other chemicals used were of reagent grade.

Enzymes. Nuclear histone acetyltransferase was purified from calf thymus according to either the procedure published earlier (Belikoff et al., 1980) or that modified as follows. Extract II after poly(ethylene glycol) fractionation was chromatographed on a DEAE-cellulose followed by a phos-

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[1] Abbreviations: CoA, coenzyme A; acetyl-CoA, acetyl coenzyme A; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride.

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phocellulose column. The kinetic results obtained with either enzyme prepartions were not altered (Garcea & Alberts, 1980).

Initial Velocity Measurements. The initial velocity of histone acetylation catalyzed by the enzyme was determined by the incorporation of the [^{3}H]acetyl group from [^{3}H]acetyl-CoA into histones, as previously described (Wong, 1980). The reaction mixture (80 μL) containing 8 μmol of Tris, pH 7.8, 2.5 μmol of potassium phosphate, pH 7.0, 0.5 μ mol of 2-mercaptoethanol, 160 μg of bovine serum albumin, varying concentrations of calf thymus histones, and [^{3}H]acetyl-CoA as indicated in the figure legends was incubated with properly diluted enzyme at 37 °C for 20 min. After that time 40 μL of the reaction mixture was spotted onto a 1-in.^{2} phosphocellulose paper (Whatman P-81) (Horiuchi & Fujimoto, 1975). The paper was then washed in 50 mM $NaHCO_3$, pH 9.0, for 15 min followed by 95% ethanol for 5 min. After the paper was dried in the oven, it was counted by using toluene scintillation fluid in a Beckman liquid scintillation counter to determine the amount of radioactivity incorporated into the histones. Control experiments were always carried out without the enzyme. Each kinetic experiment was repeated 3 or 4 times.

Analysis of Kinetic Data. Reaction velocity data were first analyzed graphically by using primary plots of reciprocal initial velocities vs. reciprocal substrate concentrations. Slopes and intercepts obtained from the primary plots were then graphed as secondary plots against either inhibitor concentrations or reciprocals of the cosubstrate concentrations. The form of the overall rate equation was determined by examination of the results of graphical analysis. Final values for the steady-state kinetic constants were obtained by fitting to eq 1 for the ping-pong initial rate pattern, to eq 2 for linear competitive inhibition, and to eq 3 for linear noncompetitive inhibition (Cleland, 1963a, 1967). The definition and notations of the kinetic constants used are those of Cleland (Cleland, 1963b).

$$v = \frac{V[A][B]}{K_a[B] + K_b[A] + [A][B]} \quad (1)$$

$$v = \frac{V[A]}{K(1 + [I]/K_{is}) + [A]} \quad (2)$$

$$v = \frac{V[A]}{K(1 + [I]/K_{is}) + [A](1 + [I]/K_{ii})} \quad (3)$$

Enzyme Intermediate Isolation. The procedure for the isolation of an acetyl-enzyme intermediate is described under the legend of Figure 4.

Results

Initial Velocity Studies. At low substrate concentrations, plots of reciprocal velocities against the reciprocal concentrations of one substrate at various fixed concentrations of the second substrate were families of parallel lines as shown in Figure 1. At high calf thymus histone concentrations, however, nonlinearity is observed, which indicates substantial inhibition at low acetyl-CoA concentrations (Figure 1A). By use of the linear region of the plot at low histone concentrations, the primary data were fitted to steady-state eq 1. Secondary plots of the intercepts from the primary data with respect to reciprocal concentrations of the fixed substrates were also linear as depicted by the inserts of Figure 1. The apparent Michaelis–Menten constants for acetyl-CoA and histones determined from these plots are respectively 1.4 ± 0.4 μM and 0.19 ± 0.03 mg/mL. The standard deviations reflect the average of three independent experiments. Similar kinetic

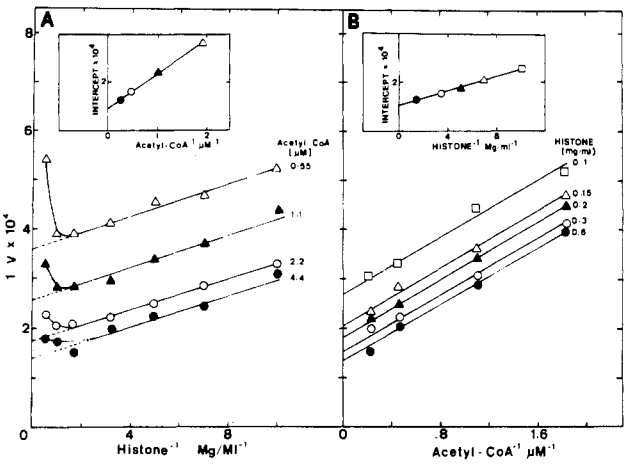


FIGURE 1: Initial velocity patterns for the histone acetyltransferase A catalyzed reaction. (A) reciprocal velocities are plotted against the reciprocal concentrations of calf thymus histones at various fixed concentrations of acetyl-CoA as indicated. (B) Reciprocal velocities are plotted against the reciprocal concentrations of acetyl-CoA at various fixed concentrations of calf thymus histones as indicated. Velocity units are counts per minute of rdioactivity incorporated as described under Materials and Methods. (Inserts) Secondary plots of intercepts from the primary plots vs. the reciprocal substrate concentrations.

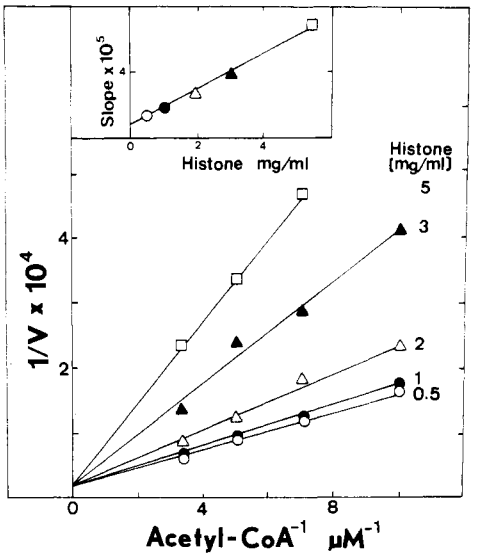


FIGURE 2: Competitive inhibition of calf thymus histones against acetyl-CoA. Reciprocal velocities were plotted against the reciprocal concentrations of acetyl-CoA at various fixed concentrations of calf thymus histones as indicated. Velocity units are counts per minute of radioactivity incorporated as described under Materials and Methods. (Insert) Secondary plot of slopes against the concentrations of histones.

patterns were observed when sea urchin sperm histones were used as a substrate, except that no inhibition was detected at high concentrations (data not shown). Moreover, a lower apparent K_m was obtained as indicated earlier (Wong, 1980). Such differences may be due to the extent of acetylation of the histones as isolated.

Histone Inhibition. Substrate inhibition was not observed with acetyl-CoA up to 7.5 μM, over 5 times its K_m. However, prominent inhibition was observed with high levels of calf thymus histones, particularly at low acetyl-CoA concentrations (Figure 1A). The inhibition when analyzed as a function of reciprocal concentrations of acetyl-CoA at fixed concentrations of histones resulted in a competitive kinetic pattern (Figure 2). By use of eq 2 and a secondary plot of the slopes from the primary data against the concentrations of histones, an

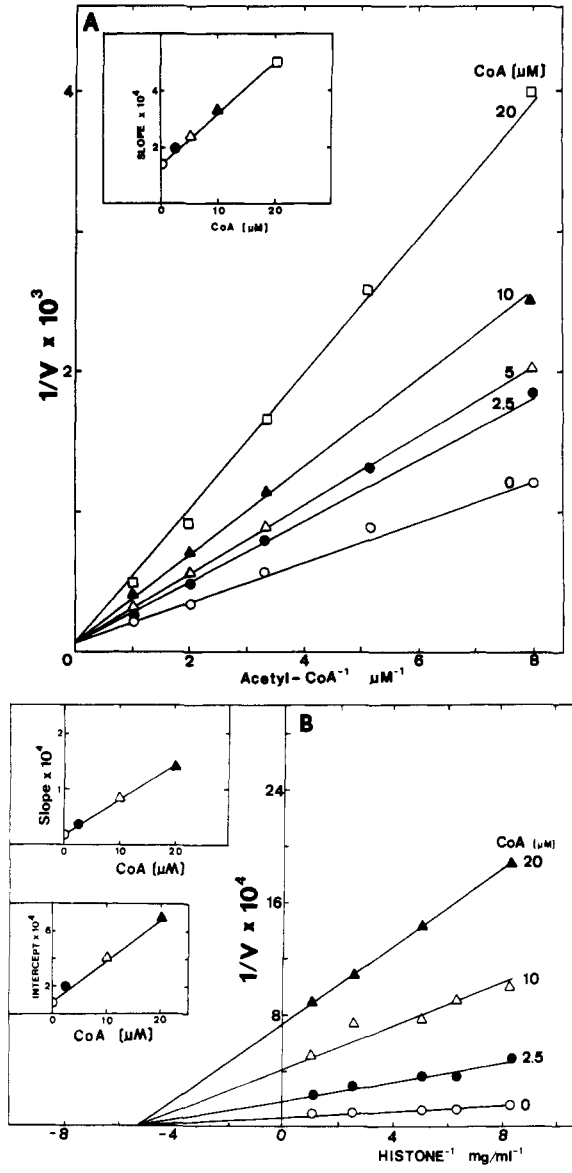


FIGURE 3: Product inhibition patterns caused by CoA. (A) Inhibition by CoA with acetyl-CoA as the varied substrate. (B) Inhibition by CoA with calf thymus histones as the varied substrate. Velocity units are counts per minute of radioactivity incorporated as described under Materials and Methods. (Inserts) Secondary plots of either the slope or intercept vs. the concentrations of CoA.

inhibition constant of 0.7 ± 0.1 mg/mL was calculated for the calf thymus histones.

Product Inhibition. Inhibition of the histone acetyltransferase A catalyzed reaction was observed when CoA was present. As depicted in Figure 3A, inhibition by CoA produced a competitive pattern with acetyl-coA as the varied substrate. However, a noncompetitive pattern was obtained with histones as the varied substrate (Figure 3B). The apparent inhibition constants for CoA were determined by a fit of the results of parts A and B of Figure 3 to eq 2 and 3, respectively, as depicted by the inserts in these figures. When acetyl-CoA was used as the varied substrate (Figure 3A), the apparent inhibition constant for CoA was calculated to be 7.5 ± 0.5 μM. From the noncompetitive pattern of Figure 3B, the apparent inhibition constants, K_{is} and K_{ii}, as calculated by eq 3, were both 3.5 ± 0.5 μM.

Isolation of Acetyl-Enzyme Intermediate. When histone acetyltransferase A was incubated with [^{3}H]acetyl-CoA in the absence of histones, a radioactive fraction containing acetyltransferase activity was isolated as shown in Figure 4A.

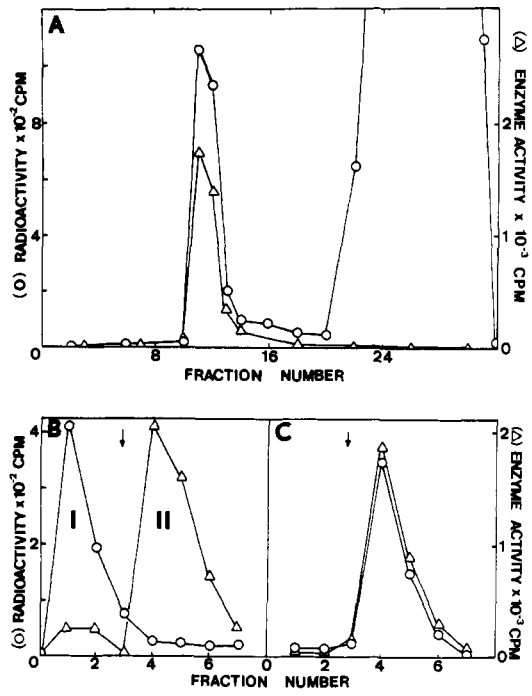


FIGURE 4: Chemical competence of an isolated enzyme intermediate. (A) Isolation of the acetyl-enzyme intermediate. Histone acetyltransferase A was incubated with 0.2 μM [^{3}H]acetyl-CoA in the absence of histones at ambient temperature for 2 min. The reaction mixture was then chromatographed at 4 °C through a 1 × 46 cm column of Sephadex G-50 equilibrated with 10 mM Tris, pH 7.8, containing 2 mM EDTA, 5 mM 2-mercaptoethanol, 20 mM NaCl, 1 mM PMSF, and 10% glycerol (buffer C). The flow rate was 12 mL/h, and 1.3-mL fractions were collected. The enzyme activity was detected as described under Materials and Methods. (B) Removal of radioactivity from isolated acetyltransferase A intermediate. Calf thymus histones were added to the enzyme fractions isolated from (A) to a final concentration of 0.1 mg/mL and incubated at ambient temperature for 2 min. The reaction mixture was then chromatographed on a 1-mL DEAE-cellulose column equilibrated with buffer C at 4 °C. The enzyme (peak II) was eluted with 0.15 M NaCl in the equilibrating buffer (↓). The radioactive peak (peak I) corresponded to the calf thymus histone elution position when it was chromatographed alone on the column. (C) A control experiment of (B). The experiment was carried out in the same manner as (B) except that no histones were added. The arrow (↓) indicates the start of elution with 0.15 M NaCl.

The radioactivity of the enzyme fraction was removed from the enzyme activity on incubation with histones followed by rechromatography on DEAE-cellulose. The radioactivity coeluted with the histone fractions (Figure 4B). In the absence of histones, however, the radioactivity remained associated with the enzymatic activity when rechromatographed on DEAE-cellulose as revealed in Figure 4C. These data indicated that an enzyme intermediate was formed on incubation with acetyl-CoA and that it was chemically competent.

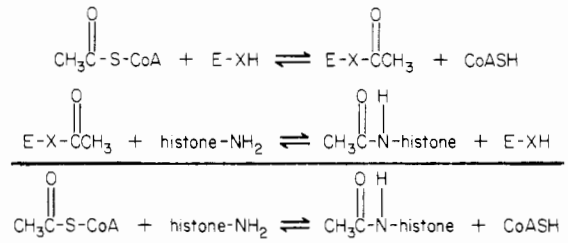
Discussion

An enzyme-catalyzed group transfer reaction can proceed via two alternative mechanisms. The first is a direct transfer of the group between the substrates at the enzyme active site in a ternary complex. The second is a double-displacement reaction involving a modified enzyme intermediate. At low substrate concentrations, the initial velocity patterns of the relationship between acetyl-CoA and histones, from either calf thymus or sea urchin sperms, for the reaction catalyzed by the histone acetyltransferase A from calf thymus are families of parallel lines (Figure 1). These results show that the reaction obeys the steady-state rate equation

$$1/v = 1/V(K_a/[A] + K_b/[B] + 1) \quad (4)$$

This is the rate equation derived for a standard ping-pong mechanism (eq 1). Thus, the observed kinetic pathway is different from that reported by Wiktorowicz et al. (1981) for the enzyme from rat liver which was suggested to follow a rapid equilibrium ordered bireactant mechanism. From such a kinetic pathway, the initial velocity pattern between acetyl-CoA and histones would be expected to be noncompetitive. For if the formation of a ternary complex containing both substrates bound simultaneously to the enzyme is a required intermediate in the reaction, the term $K_aK_b/[A]$ [B] would be added to the terms in the parentheses of eq 4, and the initial velocity pattern would be a family of lines intersecting to the left of the vertical axis (Cleland, 1963b). This is not observed in our experiments. If the formation of a ternary complex is not required but makes a significant contribution to the kinetic behavior of the reaction, a rate equation containing exponential functions of substrate concentrations would result (Wong & Hanes, 1962). The presence of exponential functions will be reflected in initial velocity patterns as nonlinear plots. With the exception of high concentrations of calf thymus histones, none of the observed kinetic data show any pattern of nonlinearity.

The ping-pong mechanism indicated by the initial velocity patterns is substantiated by the isolation of a radioactive enzyme intermediate when acetyltransferase A was incubated with [^{3}H]acetyl-CoA in the absence of histones (Figure 4A). The chemical competence of such an intermediate was demonstrated by the transfer of its radioactivity to histones on incubation (Figure 4B). Since it is the acetyl group that is being transferred from acetyl-CoA to histones, the intermediate is probably an [^{3}H]acetyl-enzyme. Thus, the results of these chemical studies support the following double displacement pathway:



While the pieces of evidence discussed above were consistent with a standard ping-pong mechanism, the inhibition patterns obtained were not. In the classical ping-pong mechanism, acetyl-CoA and acetylhistones bind to a common site on the free enzyme form, while CoA and histones also bind to this same common site but with a modified enzyme form, the acetyl-enzyme. Consequently, the product inhibition patterns between either acetyl-CoA and acetylhistones or CoA and histones will be competitive and that between either acetyl-CoA and CoA or histones and acetylhistones will be noncompetitive. However, the exactly opposite sets of inhibition patterns were observed (Figure 3): Acetyl-CoA and CoA were competitive (Figure 3A), while CoA and histones were noncompetitive (Figure 3B). These results are consistent with a two-site "hybrid ping-pong" mechanism (Northrop, 1969), with acetyl-CoA and CoA binding to one site and histones and acetylhistones binding to another independent site. Thus, the product can be released before or after the addition of the second substrate, and the reaction is not restricted either to an exclusive formation of binary complexes or to a compulsory formation of a central ternary complex. The involvement of two substrate binding sites is an obvious result of the differences between the substrates in either geometry, size, or charge (Northrop, 1969; Wong & Rose, 1976; Rose et al., 1977; Chang et al., 1983). This is consistent with the substrate specificity of enzymes. In the case of acetyltransferase A, the substrates, acetyl-CoA and histones, are distinctly different, both in charge and in structure. Thus, separate binding sites are expected. In classical ping-pong reaction pathways, the substrates are very closely related in structure (Wong & Frey, 1974a; Wong & Frey, 1974b).

Recently Cullis et al. (1982) have reported that the multisubstrate inhibitor, *N*-[2-(*S*-coenzyme A)acetyl]spermidine amide, formed by joining coenzyme A with spermidine, binds to acetyltransferase A stronger than either acetyl-CoA or histones with a K_i less than 10^{-8} M. Presumably such a strong affinity for the enzyme could arise from the interaction of the inhibitor with both the acetyl-CoA and histone binding domains of the active site.

Our studies with high substrate concentrations also support the above assertion that a hybrid ping-pong mechanism occurs with this enzyme. In the classical ping-pong pathway, substrate inhibition arises by a binding of the inhibitory substrate to the wrong form of the enzyme. Since substrates bind to a common site, the binding of the inhibitory substrate to the wrong enzyme form must compete with the binding of the second substrate, resulting in a competitive inhibition pattern. In the two-site mechanism proposed for acetyltransferase A, the substrates bind independently to different sites on the enzyme. Consequently substrate inhibition due to the binding of a substrate to the wrong enzyme form is not found. In compliance with this prediction, high acetyl-CoA concentrations were not found to be inhibitory. Similar results were obtained when sea urchin sperm histones which did not contain any acetyl group (Easton & Chalkley, 1972) were used as a substrate. No inhibition was observed at concentrations up to 0.9 mg/mL which is over 10 times its K_m (Wong, 1980). However, a competitive inhibition pattern was observed for high concentrations of calf thymus histones (Figure 2). Since the histones isolated from calf thymus are already acetylated and methylated to some degree (DeLange & Smith, 1975), and unacetylated sea urchin sperm histones do not inhibit, this seemingly conflicting observation must be due to the inhibition by the acetylated histones rather than the unacetylated histones in the substrate. The fact that unacetylated histones do not give inhibition suggests that it is the acetyl group in the partially acetylated calf thymus histones that compete with the [^{3}H]acetyl group of [^{3}H]acetyl-CoA. If acetylhistones can acetylate the enzyme, as it should for an acetyl-enzyme intermediate, the enzyme would be increasingly in the acetyl-enzyme form at increasing acetylhistone concentrations at the expense of [^{3}H]acetyl-enzyme, which is the form required to produce [^{3}H]acetylhistones followed in the assay. That is, acetylation of the enzyme by acetylhistones prevents the acetylation by [^{3}H]acetyl-CoA. Such competitive acetylation of the enzyme by acetylhistones and [^{3}H]acetyl-CoA will give rise to the observed kinetic pattern and thus render further support to the existence of an acetyl-enzyme intermediate. Because of the presence of partially acetylated histones in the calf thymus histone preparations, the determination of the Michaelis–Menten constants from Figure 1 is complicated by product inhibition. This is also evidenced in the K_m values which are higher for the histones isolated from calf thymus than those from sea urchin sperm (Wong, 1980).

Our reaction mechanism proposed for nuclear histone acetyltransferase from calf thymus differs from that proposed by Wiktorowicz et al. (1981) for the enzyme isolated from rat liver nuclei. While we do not know the source of this discrepancy, it is possible that these two enzymes are different

and that they follow different kinetic pathways. For example, we found that N^{ϵ}-acetyllysine up to 40 mM was not inhibitory to the calf thymus enzyme whereas it was a good inhibitor for the rat liver enzyme (Wiktorowicz et al., 1981).

During the course of our kinetic studies, we have paid particular attention to our control experiments. We have found that histones undergo nonenzymatic acetylation which is a function of the acetyl-CoA concentrations. Such noncatalytic incorporation of acetyl group may give rise to different initial velocity patterns, if it is not corrected. In addition we have used a large range of substrate concentrations. This is particularly important in the case where substrate inhibition is involved. Thus, for acetyltransferase A we have found that within a narrow high concentration range of histones, its inhibition may be misinterpreted as a different initial velocity pattern (Wiktorowicz et al., 1981).

The proposed mechanism for acetyltransferase A from calf thymus is consistent with all the data reported here and combines the features of a substituted enzyme intermediate with an independent binding of substrates, which is a characteristic of the random, ternary complex mechanism. We are currently investigating the properties of the acetyl-enzyme to identify the nucleophilic group at the active site of the enzyme.

Registry No. Acetyl-CoA, 72-89-9; CoA, 85-61-0; EC 2.3.1.48, 9054-51-7.

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