Ubiquitin Carrier Protein-catalyzed Ubiquitin Transfer to Histones

MECHANISM AND SPECIFICITY*

(Received for publication, March 17, 1988)

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Ubiquitinated derivatives of histones H2A and H2B, in which the carboxyl terminus of ubiquitin is joined to ε-amino groups of specific lysine residues of each histone, occur in vivo. Certain ubiquitin carrier proteins (E2s) catalyze ubiquitin transfer to histones (Pickart, C. M., and Rose, I. A. (1985) J. Biol. Chem. 260, 1573–1581). The catalytic activities of these purified ubiquitin carrier proteins have been quantitatively characterized with purified histones, in order to determine if one or more of them exhibits specificity for H2A over other histones (H3;H4) which are not known to be ubiquitinated in vivo. The results show the following. 1) No E2 exhibits strong specificity for H2A over the other histones. 2) For a given histone, kinetics of formation of its monoubiquitinated adduct do not differ strongly among the E2s; sigmoid kinetics (n_H = 2) are generally observed, with values of K_m ranging from 2–6 μM. 3) E214K catalyzes primarily monoubiquitination. 4) E220K catalyzes multiple ubiquitination (up to three ubiquitin/histone) by a processive mechanism that involves joining of ubiquitin carboxyl termini to multiple histone lysine residues. 5) E220K also catalyzes processive ubiquitination, with formation of polyubiquitinated products exhibiting a lag phase. Many of the polyubiquitinated adducts produced at low histone concentration are expected for monoubiquitination of every histone-lysine residue, and polyubiquitination is selectively inhibited by substitution of reductively methylated ubiquitin for ubiquitin. These results suggest that E220K uniquely catalyzes ubiquitin transfer to lysine residues of previously-conjugated ubiquitin molecule(s). The implications of these results for biological mechanisms of histone ubiquitination are discussed.

The small protein ubiquitin is the most highly conserved protein currently known in eukaryotes (1). Within cells, ubiquitin is found in two forms, as the free polypeptide (molecular mass 8.5 kDa), and covalently joined to a variety of cellular proteins (2–4). Ubiquitin-protein conjugation occurs through formation of amide bonds between carboxyl-terminal glycine residues of ubiquitin molecules and (usually) ε-amino groups of cellular proteins (3, 5). Ubiquitin-protein conjugates typically comprise about 50% of total cellular ubiquitin (6), and occur in locations that include cell surface (7), cytoplasm (4), mitochondria (4), and nucleus (2). Many cytoplasmic conjugates, and some of those in other locations (e.g. mitochondria) accessible to enzymes in the cytoplasm, appear to be intermediates in a multienzyme, ATP-dependent proteolytic pathway. Selective conjugation to ubiquitin renders short-lived and abnormal proteins susceptible to degradation by a complex, ATP-dependent protease (8–10).

The nuclear histone conjugates uH2A and UH2B account for about 10 and 1% of their respective histone pools (11, 12). In each adduct, 1 mol of ubiquitin/mol of histone is ligated to the ε-amino group of a specific lysine residue (2, 13). The physiological roles of histone-ubiquitin conjugation are incompletely understood. Levels of uH2A fall during mitosis (14, 15); the activity of a ubiquitin-histone isopeptidase (16) may contribute to this cell-cycle-correlated change. Chromatin of some actively transcribed genes appears to be enriched in ubiquitinated histones (17, 18), while chromatin of another actively transcribed gene is reported to be depleted of ubiquitinated histones (19). These results suggest that ubiquitination may influence (or be influenced by) chromatin condensation state and transcriptional activity under some conditions.

The formation of cytoplasmic conjugates is a three-step process that begins with formation of a thiol ester bond between the ubiquitin carboxyl terminus and a thiol group of an ubiquitin carrier protein or E2 (Equation 1). This is followed by ubiquitin transmigration to a carrier protein or E2 (Equation 2). In the formation of conjugates fated for degradation, transfer of ubiquitin (Ub) from E2-ubiquitin to protein substrate is catalyzed by a ligase or E3 (Equation 3) (PP_n, pyrophosphate) (20).

\[
\begin{align*}
O & \quad \text{Mg}^+ \quad O \\
2 \text{ATP} + 2 \text{UbCo}^- + \text{E1-SH} & \quad \text{E1-SCUb} \quad \text{(1)} \\
\text{AMP-Ub} + \text{AMP} + 2 \text{PP} & \\
\text{O} & \\
\text{E1-SCUb} + \text{E2-SH} & \quad \text{E2-SCUb} + \text{E1-SH} \quad \text{(2)} \\
\text{O} & \\
\text{E2-SCUb} + \text{RNH}_2 & \quad \text{RNCUb} + \text{E2-SH} \quad \text{(3)} \\
\end{align*}
\]

Although there are multiple E2s (20, 21), only the smallest of them, E214K, appears to function in conjugation that leads to

* This research was funded by Grant DMB 8603551 from the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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The abbreviations used are: uH2A and uH2B, biologically occurring monoubiquitinated derivatives of H2A and H2B, respectively; E1, ubiquitin activating enzyme; E220K, E220K, and E214K, ubiquitin carrier proteins of subunit molecular masses 35, 20, and 14 kDa, respectively (these correspond to E2s 1, 3, and 5 in the previous nomenclature (21)); E3, ubiquitin-protein ligase; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; FPLC, fast protein liquid chromatography.
protein breakdown (21, 22). Most of the E2s are homodimers (21, 22).

Cells having a thermolabile E1 are defective in ubiquitin-protein conjugation and intracellular proteolysis at the non-permissive temperature, and arrest in G2 phase, concomitant with loss of ubiquitin from ubiquitin ligase (14). These results suggest that conjugates are intermediates in a ubiquitin-dependent degradative process and show that an active ubiquitin-protein ligation pathway is required for cell-cycle progression. They also show that the same E1 functions in formation of cytolytic and nuclear conjugates.

Subsequent steps in the formation of nuclear ubiquitin conjugates have not been completely elucidated. Several E2s catalyze ubiquitin transfer to histones in vitro. In the reactions catalyzed by E2A4, E2A9, and E2A5, the predominant product seen with H2A (14 \mu M) is a monoubiquitinated adduct; the other E2s are inactive in ubiquitin transfer to histones (22). Whether ubiquitination occurs at a specific H2A lysine residue is unknown. The possible function of one or more of these enzymes in biological histone ubiquitination is of particular interest in light of the recent demonstration that yeast E2A5 is identical to the DNA repair enzyme RAD6 (23).

We have shown previously that E2A4, E2A9, and E2A5 catalyze ubiquitin transfer to a histone (H3) that is not known to be ubiquitinated in vivo (22). It was therefore of interest to determine whether one or any of these enzymes catalyzes ubiquitin transfer to a physiological substrate, H2A, more efficiently than to other histones that are not known to be physiological substrates (H3, H4).

**EXPERIMENTAL PROCEDURES**

**Materials**—Ubiquitin used in enzymatic assays was prepared from outdated human blood (24). Reductively methylated ubiquitin was prepared as described previously (25). Assay with fluorescamine (26) showed that reductive methylation blocked >98% of the free amino groups of ubiquitin. Ubiquitin and reductively methylated ubiquitin were radiodinated using chloramine T (3) (initial specific radioactivities, 8000 and 600 cpm/pmole, respectively). Ubiquitin-Sepharose affinity resin, 5 mg ubiquitin/ml resin, was prepared (20) from bovine ubiquitin (Sigma) and activated CH-Sepharose (Pharmacia LKB Biotechnology Inc.). Carrier-free iodine-125 was from Amersham Corp.; [\gamma-32P]ATP (3000 C/mmol) was from Du Pont-New England Nuclear.

**Enzyme Preparations**—Fraction II was prepared from rabbit reticulocytes (reticulolysis induced with phenylhydrazine) as described previously (20). E1 and E2s were prepared from fraction II by a modification of published procedures that involves prefractionation of the fraction II (22). This method selectively removes ubiquitin-protein ligase activity prior to ubiquitin affinity chromatography and improves recovery of E2s. Briefly, fraction I (360 mg) was applied to a 20-ml column of Q-Sepharose fast flow (Pharmacia LKB Biotechnology Inc.) previously equilibrated with 50 mM Tris-HCl (6% base), 0.5 mM EDTA, pH 7.5 at 5 °C. After washing with 20 ml of equilibration buffer, the column was eluted sequentially with 40-ml portions of 40 mM Tris-HCl (10% base), 0.1 mM EDTA, 0.5 mM EDTA (5 °C). Fraction C, which contained E1, E2A9, and a high-molecular-weight E2 (22), was concentrated to about 2 ml (Centricon-10, Amicon) and dialyzed overnight against 20 mM Tris-HCl (10% base), 0.1 mM EDTA, 0.5 mM EDTA, 0.2 mg/ml ovalbumin (5 °C). Fractions of 1 ml were collected; E1 (100 kDa) and E2A9 (25 kDa) eluted in fractions 26-38. These fractions were pooled, concentrated, and subjected to ubiquitin affinity chromatography (below). This gel filtration step largely removed a high molecular weight ligase activity from fraction C.\(^2\)

\(^2\) N. Schneider, A. Vella, and C. Pickart, unpublished observations.

As described previously, fractions C, D, and E contain E2A4, E2A9, and E2A5, respectively (22). The E2s were purified by affinity chromatography (following addition of 0.2 \mu M of purified E1 to fractions D and E). Fractions derived from 360 mg of fraction II were applied to 2-ml columns, and affinity chromatography was carried out essentially as described previously (20-22). E1 and E2 were eluted simultaneously with a buffer containing 50 mM Tris-HCl, 0.5 mM DTT, 0.1 mM EDTA, 2 mM DTT, 50 mM lysine-HCl. Following dialysis and concentration of the eluate, E1 was resolved from E2 on an FPLC Mono Q column (LKB Biotechnology Inc. Pharmacia), as described previously (22). In the case of E2A4 and E1, which are poorly resolved, the Mono Q peak was concentrated and run on a Superose 12 high-resolution gel filtration column (Pharmacia Biotech Inc.) (22). These procedures resulted in >95% homogeneous E1 and E2 preparations, as judged by SDS-PAGE and silver staining (22). Overall recovery of E2s from fraction II was typically 30-70%.

**Concentrations of E1 and E2s**—were determined from the magnitudes of the \([\gamma-32P]P_i\) bursts observed in the presence of \([\gamma-32P]P_i\) ATP and inorganic pyrophosphatase, determined by extraction of phenol, and iodination of isobutanol as described previously (21). E1 concentration was obtained by dividing its burst by two (Equation 1); E2 concentration was assumed to be stoichiometric with the \(E2^-\) dependent burst (measured in the presence of 70 nm E1).

**RESULTS**

**Purification**—Dr. S. Matsui, Roswell Park Memorial Institute, provided a crude mixture of calf thymus histones H1, H2A, H3, and H4. The mixture was chromatographed in Bio-Gel P-60 (27), resulting in preparations of H4 and H3/H2A. H3 and H2A were resolved from one another by precipitation of H3 with ethanol (28). Histone purity was >98% in all cases, based on SDS-PAGE and analysis of Blue Staining. Histone dilutions (in solutions containing 0.2 mg/ml ovalbumin) were prepared freshly for each experiment from 2-5 mg/ml stock solutions.

**Assay of Histone Ubiquitination**—Incubations (10 \mu l) contained 50 mM Tris-HCl (24% base), 5 mM MgCl2, 2 mM ATP, 0.6 \mu M inorganic pyrophosphatase, 10 mM creatine phosphate, 3 units/ml creatine kinase, 2-4 \mu M radioiodinated ubiquitin, 0.3 mg/ml ovalbumin, 0.2 mM DTT, histones at concentrations indicated in the figure legends, 100 mM E1, and 14-120 mM E2, pH 7.3, 37 °C. After 5, 10, or 15 min, 10 \mu l of SDS-PAGE sample buffer was added to quench the reaction, and it was heated to 100 °C for 1 min. An aliquot was electrophoresed, and the gel dried and autoradiographed (below). To quantitate products, appropriate radioactive bands were excised from the dried gel and counted in a gamma counter (LKB Minigamma). Observed counts/minute were corrected by subtracting counts/minute in analogous slices derived from incubations lacking E2. Background counts/minute were generally <10% of experimental counts/minute.

For measurement of ATP hydrolysis during histone ubiquitination, assays (40 \mu l, pH 7.3, 37 °C) contained 50 mM Tris-HCl (24% base), 5 mM MgCl2, 20 \mu M \([\gamma-32P]P_i\) ATP (500 cpm/pmole), 0.6 \mu M inorganic pyrophosphatase, about 0.3 mg/ml ovalbumin, 0.1 mM DTT, 100 mM E1, 50-120 mM E2, and various histone concentrations. Counts/minute at 10 min were removed at 10, 20, and 30 min, and assayed for \([\gamma-32P]P_i\) (21).

**Electrophoretic Methods**—SDS-PAGE was carried out using a mini-gel apparatus (Bio-Rad) in gels of 12.5% acrylamide, according to the method of Laemmli (29). SDS-PAGE sample buffer contained 50 mM Tris-HCl, pH 6.8, 4% SDS, 20% (v/v) glycerol, 0.4% 2-mercaptoethanol. In some cases, molecular weight standards were run to estimate the sizes of the ubiquitin-histone adducts, and gels were stained with Coomassie Blue. Dried gels were autoradiographed at -60 °C using Du Pont Cronex intensifying screens and Kodak XAR-5 film.

**RESULTS**

Product distributions and rates of product formation were measured as a function of concentrations of H3 (15.5 kDa), H2A (14 kDa), and H4 (12 kDa) (30), for each of the three E2s that catalyze ubiquitin transfer to histones (E2A4, E2A9, and E2A5). E2A4 and E2A9,Refs. 21-23). Appropriate controls involving each of these enzymes showed the following: 1) Negligible histone conjugation occurred in the absence of E2. 2) At 12.9 \mu M H3, doubling E2 concentration doubled the total rate of histone conjugation, based on measurement of conjugated radiolabeled ubiquitin. The rate was unaffected by doubling E1.
3) At 12.9 μM H3, the rate of [32P]pyrophosphate production (evaluated between 10 and 30 min) was equal to the total rate of formation of radioiiodinated adducts (evaluated between 5 and 15 min), within experimental error. This was true for each E2. 4) In all assays, the fraction of histone that became conjugated to ubiquitin was <10% of that present in the assay; the fraction of ubiquitin that became conjugated to histone was <15% of that (2–4 μM) present in the assay. 5) Dilute solutions of E235~ (0.6 μM, but containing carrier ovalbumin) were quite labile to freezing and thawing. All experiments involving E235~ were done with freshly diluted enzyme. 6) Rates of ubiquitination tended to decrease upon extended incubation (>30 min), possibly as a result of enzyme inactivation.

Histone Ubiquitination Catalyzed by E214K—The radiolabeled products formed during a 10-min incubation of E214K with radioliabeled ubiquitin and H2A (together with E1 and MgATP) are shown in Fig. 1A. The molecular mass of the single major product, 21.5 kDa, suggests that it contains 1 mol each of ubiquitin (8.5 kDa) and H2A (14 kDa). Higher molecular weight products, presumably resulting from transfer of two or three ubiquitin/H2A, were visible upon much longer exposure of the autoradiograph (not shown). In contrast to results obtained with the other E2s (below), however, these higher order products were minor at all H2A concentrations.

Linearity of product formation with time is shown in Fig. 1B for H3, and was assumed to hold for other histones as well. Therefore single time point assays such as those shown in Fig. 1A are measures of initial rates. The dependence of the initial rate of H2A conjugation on H2A concentration is shown in Fig. 1C. The rates are expressed relative to the rate at 14.3 μM H2A (Vmax); this simplified comparison of data obtained in experiments at slightly different E2 concentrations. For H2A and H3, rates were unchanged between 13 and 50 μM histone (not shown).

The concentration dependence for H2A is sigmoidal (inset, Fig. 1C). This behavior was unaffected by enzyme concentration: at all substrate concentrations, rates were directly proportional to E214K concentration, over an 8.5-fold range (not shown). This result suggests that sigmoidicity is not an artifact resulting from incomplete saturation with E1, or from histone absorption. (Ovalbumin, 0.3 mg/ml, was present in all assays to counteract absorption of histone and enzymes.) The line in Fig. 1C is calculated for values of K0.5 = 3.2 μM and a Hill coefficient (nH) of 2.0 (Table I).

A monoubiquitinated adduct (23 kDa) is also the predominant product of catalytic ubiquitin transfer to H3 (Fig. 1A), and the concentration dependence is sigmoidal (Fig. 1D; Table I). The dependence of the rate of formation of the monoubiquitinated adduct of H4 (18.5 kDa) on H4 concentration also appears to be sigmoidal (Fig. 1E), but the value of nH is uncertain because the highest H4 concentration, 16.7 μM, may not have been saturating. Consequently only a lower limit can be placed on the value of K0.5 for H4 (≥6 μM, Fig. 1D). Kinetic constants for E214K catalysis are summarized in Table I.

![Fig. 1. E214K-catalyzed histone ubiquitination. A, product size and distribution (autoradiograph). Assays were carried out for 10 min as described under "Experimental Procedures." Lanes 1 and 2, H2A was present at concentrations of 2.9 and 7.1 μM, respectively; lane 3, H3 (12.9 μM); lane 4, H4 (16.7 μM). Arrowheads denote monoubiquitinated histone adducts. The designation top indicates the top of the resolving gel; Ub, free ubiquitin band. B, time course of H3 ubiquitination (12.9 μM H3, 120 nM E2). C–E, histone concentration dependence of ubiquitination rate, based on 10- or 15-min assays (C, H2A; D, H3; E, H4). Each panel combines the results of two separate experiments (in panel E, [E2] was 120 nM). The inset in panel C shows a Hill plot of the data for H2A (R = (100 – %Vmax)/%Vmax). Lines in panels C and D are calculated from the parameters shown in Table I; the line in panel E has no theoretical basis.](image-url)
Kinetic parameters for formation of monoubiquitinated histone adducts

| E2   | Substrate | n_{st} | K_{on} | k_{cat} |
|------|-----------|--------|--------|---------|
| E2_{20K} | H4       | 2.0    | 3.9    | 0.09    |
|       | H3       | 1.7    | 3.5    | 0.06    |
|       | H2A      | 1.0    | 2.3    | 0.06    |
| E2_{20K} | H4       | 2.5    | 2.4    | 0.18    |
|       | H3       | 2.0    | 1.8    | 0.10    |
|       | H2A      | 2.0    | 1.9    | 0.14    |
| E2_{14K} | H4       | -      | 6.0    | 0.09    |
|       | H3       | 2.2    | 3.0    | 0.07    |
|       | H2A      | 2.0    | 3.2    | 0.06    |

* Although concentration dependence probably sigmoidal, value of n_{st} is not known (text; Fig. 1E).

Histone Ubiquitination Catalyzed by E2_{20K}—E2_{20K} catalyzed ubiquitin transfer to H2A gives several prominent products of molecular masses consistent with conjugation of one (21.5 kDa), two (29.5 kDa), or three (36 kDa) ubiquitin, respectively, per H2A (Fig. 2A). There is also a minor product slightly larger than the putative n = 2 adduct that may result from conjugation of a slight H3 contaminant (Fig. 2A, lane 1). With H2A, H3, and H4, the distribution of monoubiquitinated and multiply ubiquitinated products was unchanged if reductively methylated ubiquitin was substituted for normal ubiquitin; this is shown for H4 in Fig. 3 (lanes 1 and 2). Therefore, multiply ubiquitinated products result from conjugation of ubiquitin carboxyl termini to histone lysine residues, and not conjugation of secondary ubiquitin molecules to lysine residues of previously conjugated ubiquitin. Such “ubiquitin-ubiquitin” conjugation has been shown to occur under some conditions (Ref. 25 and below).

As shown in Fig. 2B for H3, formation of products with n = 1, n = 2, and n = 3 stoichiometries is linear with time at 12.9 μM H3; the same result was obtained at 2.6 μM H3 (not shown). This is the result expected if formation of multiply ubiquitinated products occurs through a processive mechanism in which several ubiquitin transfer events occur prior to product dissociation. However, the distribution of multiply ubiquitinated products changes as H2A concentration increases, with higher order products becoming progressively less abundant; this behavior is more pronounced with H3 and H4 (Fig. 2A). At very high H2A concentration (70 μM), only the n = 1 product is observed (not shown). This result is not expected for a simple kinetically processive mechanism, in which the partition of bound substrate between further ubiquitination and dissociation should be independent of substrate concentration.

The dependence of the initial rate of formation of monoubiquitinated H2A on H2A concentration is sigmoidal (Fig. 2C; Table 1). Similar results were obtained with the substrates H3 and H4. For H3, products with molecular masses of 24, 31.5, and 38.5 kDa are consistent with conjugation of one, two, or three ubiquitin, respectively, per H3. For H4, products with the same apparent stoichiometries have sizes of 18, 27, and 34 kDa. With both substrates, formation of higher molecular weight products is selectively inhibited at high histone concentration (Fig. 2A), and the dependence of the rate of formation of monoubiquitinated histone on histone concentration is sigmoidal (Figs. 2D and E, Table 1). For H3, the monoubiquitination rate decreased by 50% on increasing H3 concentration from 12.9 to 72 μM (not shown). Kinetic parameters for E2_{20K}-catalyzed formation of n = 1 adducts are summarized in Table 1.

Histone Ubiquitination Catalyzed by E2_{20K}—Catalysis of polyubiquitination by E2_{20K} is very dramatic at low histone concentration, as shown for H4 in Fig. 4A. At 2.6 μM H4, prominent products have molecular masses of 20, 31.5, 37, and 43 kDa; these are approximately consistent with transfer of 1–4 ubiquitin/H4. In addition, there are large “smears” at the tops of lanes 1–5 (Fig. 4A), which actually contain discrete bands (e.g. lane 5, Fig. 4A). Products of apparent stoichiometry ubiquitin/H4 > 4 comprise 78%, and the n = 1 adduct comprises 6%, of the total product formed in a 10-min incubation at 2.6 μM H4. The formation of very high molecular weight products is profoundly inhibited at H4 concentrations above 3 μM; at 16.7 μM H4, the n = 1 adduct comprises 50% of total product and ubiquitin/H4 > 4 products are virtually absent (Fig. 4A). Since there are only 11 lysine residues in calf thymus H4, monoubiquitination of every lysine residue should give a product of 100 kDa. The largest H4 conjugates are larger than this (Fig. 3, lane 3). That most E2_{20K}-catalyzed conjugation is ubiquitin-ubiquitin conjugation (25) is suggested by the profound inhibition of formation of large conjugates that occurs when reductively methylated ubiquitin is substituted for ubiquitin (Fig. 3, lanes 3–6).

Time courses of product formation during E2_{20K} catalysis at two different H3 concentrations are shown in Figs. 4, B and D. (As described below, distinctive features of E2_{20K} catalysis are virtually identical for H2A, H3, and H4.) At 12.9 μM H3, formation of the (major) n = 1 product is linear with time, but formation of n = 2 and n = 3 products shows an initial lag (Fig. 4B). At 1.3 μM H3, the formation of high molecular weight (n > 3) products also shows a lag, but these products are much more abundant than n = 1 and n = 2 products (Figs. 4, C and D). Thus, product distribution changes as a function of time, in addition to changing with substrate concentration. This suggests that high molecular weight products are formed from smaller products. That such products can be formed in the presence of a (relatively) high concentration of free histone suggests that ubiquitination of H3 increases its affinity for E2_{20K}. Comparison of lanes 1 and 2–5 in Fig. 4D also reveals subtle differences in products as a function of H3 concentration; at 1.3 μM H3, the putative n = 2 adduct is a doublet, and there is a band below the n = 1 product. The source of these “extra” bands at low H3 concentration is unknown.

The basic features of E2_{20K}-catalyzed ubiquitin transfer are similar for the substrates H4, H3, and H2A (Fig. 4A). In each case, high molecular weight products predominate at low substrate concentration, and formation of these products is highly inhibited at substrate concentrations (13–17 μM) that are saturating for formation of the n = 1 adducts (Fig. 4E–G). As in the case of H4, formation of high molecular weight products from H3 and H2A was profoundly inhibited by substitution of reductively methylated ubiquitin for normal ubiquitin (not shown). For H3, the rate of formation of the n = 1 adduct decreased by 30% on increasing H3 concentration from 12.9 to 72 μM.

The dependence of the rate of n = 1 adduct formation on substrate concentration is shown in Figs. 4E (H4), F (H3), and G (H2A). H4 and H3 show sigmoidal behavior. For H2A, the data are adequately fit assuming normal hyperbolic kinetics, although it is difficult to exclude the possibility of sigmoidal kinetics with a low value of K_{on}. Kinetic parameters for E2_{20K} catalysis are summarized in Table 1.
**Specificity in E2-catalyzed Histone Ubiquitination**

**Fig. 2.** $E_2^{20K}$-catalyzed histone ubiquitination. A, product sizes and distributions (autoradiograph). Lanes 1–5, H2A was present at concentrations of 1.4, 2.9, 4.3, 7.1, and 14.3 μM, respectively; lanes 6 and 7, H3 (2.6, 12.9 μM); lanes 8 and 9, H4 (1.7, 16.7 μM). Arrowheads denote monoubiquitinated histone adducts. *Ub*, ubiquitin. B, time course of H3 (12.9 μM) ubiquitination: formation of $n = 1$ (○), $n = 2$ (□), and $n = 3$ (●) adducts (120 nM $E_2$). C–E, histone concentration dependence of rate of formation of monoubiquitinated adduct (C, H2A; D, H3; E, H4). Each panel combines the results of two separate experiments. The line in each panel is calculated from the parameters shown in Table 1.

**Fig. 3.** Multiple ubiquitination catalyzed by $E_2^{20K}$, but not $E_2^{20G}$, is inhibited by substitution of reductively methylated ubiquitin (Ub) for native ubiquitin (autoradiograph). Lanes 1 and 2, $E_2^{20K}$ in the presence of 16.7 μM H4; lane 1, radioiodinated native ubiquitin (4 μM); lane 2, radioiodinated reductively methylated ubiquitin (3.5 μM). Lanes 3–6, $E_2^{20K}$ in the presence of 1.7 μM (lanes 3 and 4) or 16.7 μM (lanes 5 and 6) H4. Lanes 3 and 5, native ubiquitin; lanes 4 and 6, reductively methylated ubiquitin. Incubations were carried out for 10 min as described under “Experimental Procedures” (~130 nM $E_2$). Because of the lower specific radioactivity of the methylated ubiquitin, autoradiographic exposure times for lanes 2, 4, and 6 were longer than for lanes 1, 3, and 5. Arrowhead shows the $n = 1$ adduct; the asterisk denotes the position of the 116 kDa molecular mass standard (α-galactosidase).

**DISCUSSION**

Values of $k_{cat}$ for $n = 1$ adduct formation catalyzed by $E_2^{20K}$, $E_2^{20G}$ (RAD6), and $E_2^{44K}$ range between 0.05 and 0.14 min$^{-1}$ (Table 1). The $k_{cat}$ values for H3 and H2A are in reasonable agreement with those reported previously (22). Histone $k_{cat}$ values are similar to that (0.05 min$^{-1}$) for $E_2^{20K}$-catalyzed monoubiquitination of yeast cytochrome c (21). However, $K_{0.5}$ values for histone substrates of $E_2^{20K}$ (Table I) are about 20-fold smaller than the $K_m$ for yeast cytochrome c (56 μM), and cytochrome ubiquitination did not show sigmoidal kinetics (21). Our current results show that the predominance of the monoubiquitinated product seen previously (21, 22) was a result of the high histone concentration used. Only $E_2^{44K}$ catalyzes transfer of predominantly 1 mol of ubiquitin/mol histone at all histone concentrations. It remains to be determined whether this transfer occurs at a unique histone lysine residue, and whether transfer catalyzed by $E_2^{20K}$ and $E_2^{20G}$ begins at a specific histone lysine residue.

$E_2^{44K}$ typically transfers up to 3 mol of ubiquitin/mol histone (Fig. 2A). The invariance of product distribution as a function of time (Fig. 2B) is consistent with multiply ubiquitinated conjugates arising from a kinetically enforced processive mechanism in which several transfer events occur per binding event, as a result of comparable rate constants for further ubiquitination and for product dissociation. However, a change in product distribution as a function of substrate concentration (Fig. 2A) is not expected for this model. The results can be qualitatively explained if the rate constant for ubiquitin transfer decreases, or that for product dissociation increases, with increasing substrate concentration. The de-
Specificity in E2-catalyzed Histone Ubiquitination

FIG. 4. E2sar-catalyzed histone ubiquitination. A, product sizes and distributions (autoradiograph). Lanes 1–6, H4 was present at concentrations of 1.25, 1.7, 2.6, 3.4, 6.8, and 16.7 μM, respectively; lanes 7 and 8, H3 (1.3, 12.9 μM); lanes 9 and 10, H2A (1.4, 14.3 μM). Arrowheads denote monoubiquitinated histone adducts. Ub, ubiquitin. B–D, time dependence of H3 ubiquitination. B, ubiquitination at 12.9 μM H3 (120 nM E2): formation of n = 1 (O), n = 2 (□), and n = 3 (●) adducts. C, ubiquitination at 1.3 μM H3 (~80 nM E2): formation of n = 1 (O), n = 2 (□), and n = 3 (●) products. D, autoradiograph of gel from which some of the data plotted in panels B and C were derived. Lane 1, products formed in the presence of 12.9 μM H3 after 10 min of incubation. Lanes 2–5, products formed in the presence of 1.3 μM H3 after 2, 4, 6, and 10 min of incubation, respectively. E–G, histone concentration dependence of rate of formation of n = 1 adduct (E, H4; F, H3; G, H2A). Panels F and G combine data from two separate experiments. Lines in each panel are calculated from the parameters shown in Table I.

crease in kcat for formation of monoubiquitinated H3 that occurs at high H3 concentration (“Results”) is consistent with the former explanation. Alternatively, processivity could result from a change in histone affinity upon ubiquitination; if this is the case, the increase in affinity must be larger than that postulated to occur with E2sar (below), in order to account for linear rate of formation of higher order products seen with E2sar. The failure of reductively methylated ubiquitin to alter product distribution (Fig. 3) shows that multiple ubiquitination catalyzed by E2sar involves joining of ubiquitin carboxyl termini to histone lysine residues, rather than to lysine residues of previously conjugated ubiquitin.

E2sar also catalyzes multiple ubiquitination (Fig. 4A). Evidence that most of this polyubiquitination results from conjugation of ubiquitin to previously conjugated ubiquitin are the observations that 1) a significant fraction of the products seen at low histone concentration are larger than predicted for monoubiquitination of every lysine residue of the histone; and 2) substitution of reductively methylated ubiquitin for native ubiquitin strongly inhibits the formation of large conjugates (Fig. 3). Catalytic product distribution changes with time, with appearance of polyubiquitinated conjugates showing a lag phase (Fig. 4B and C). Product distribution also changes with substrate concentration: formation of highly ubiquitinated products is selectively inhibited at high substrate concentration. All of these results are qualitatively consistent with a model in which E2sar-catalyzed polyubiquitination arises from a thermodynamically processive mechanism in which ubiquitination of the histone increases its affinity for E2sar. In this model, high molecular weight products will not form until a significant concentration of small product has accumulated, so that a lag in formation of high molecular weight products might occur. As free histone concentration surpasses that required to saturate the rate of n = 1 adduct formation, product will compete increasingly less effectively with free histone, and polyubiquitination will be
selectively inhibited. This model can be tested experimentally.

The occurrence of multiply ubiquitinated histones in vivo has recently been reported; the level of diubiquitinated H2B was found to increase during trout testis development (31). Polyubiquitination was shown to involve conjugation of ubiquitin to lysine residues (31), but it was not determined whether these were histone or ubiquitin lysine residues. Our results suggest that, depending on the nature(s) of the linkages of ubiquitin molecules, E2

\[ k_{\text{cat}} \] and/or E2

\[ K_{\text{m}} \] could participate in biological polyubiquitination.

For a given E2, there is little difference in catalytic properties as a function of histone identity (Table I, Figs. 1, 2, and 4). In addition, \( k_{\text{cat}} \) and \( K_{\text{m}} \) values for a particular histone do not vary strongly between E2s (Table I). The generally sigmoidal kinetics could arise if substrate binding to one subunit of the dimeric E2 increased the substrate affinity of the other subunit, or possibly from concentration-dependent histone-histone interactions. In the case of E2

\[ k_{\text{cat}} \] is possible that observed rates (of \( n = 1 \) adduct formation) at low substrate concentration may underestimate the true rate of this reaction, because the \( n = 1 \) product may be consumed as a substrate for further ubiquitination (above). This behavior could lead to artificial sigmoidal kinetics and to overestimation of the value of \( K_{\text{m}} \). However, sigmoidal kinetics were observed with most substrates in the reactions catalyzed by the other two enzymes, where this consideration does not apply. E2

\[ k_{\text{cat}} \] does show several differences from E2

\[ k_{\text{cat}} \] and E2

\[ k_{\text{cat}} \]. 1) \( k_{\text{cat}} \) values are larger, and \( K_{\text{m}} \) values are smaller, by about 2-fold. 2) The order of histone reactivity (\( k_{\text{cat}} \)) is different (H2A > H3, H4, versus H3 > H2A, H4).

Although there are certain E2-specific mechanistic features in catalysis of ubiquitin transfer to histones, the results do not strongly implicate one of these enzymes (versus the others) in biological histone ubiquitination. Each enzyme catalyzes ubiquitin transfer to histones not known to be ubiquitinated in vivo, with kinetics similar to those of the physiological substrate H2A. Catalysis of ubiquitin transfer to H2B was demonstrated previously with an E2 mixture (21); there is little reason to expect that the quantitative substrate properties of H2B with individual E2s would differ strongly from those described here for other histones.

Several mechanisms could operate in cells to modulate the apparent lack of specificity in E2-catalyzed histone ubiquitination. One possibility is that histones contained within nucleosomes are the predominant substrates for ubiquitination. The presence of a histone in a nucleosome is likely to mask some ubiquitin acceptor sites, and histone assembly into nucleosomes are the predominant substrates for ubiquitination. Some ubiquitin acceptor sites, and histone assembly into nucleosomes may occur in the cytoplasm, prior to histone import into the nucleus (32). A second possibility is that a specific role for one or more E2s in biological histone ubiquitination might be enforced through differences in intracellular localization of the E2s. While the nuclear membrane is permeable to many small proteins, proteins larger than about 60 kDa appear to be unable to cross the nuclear membrane unless they contain within their sequences a specific uptake signal (33, 34). Thus E1 (100 kDa, Ref. 21) and E2

\[ K_{\text{m}} \] could be excluded from the nucleus based on size. A nuclear localization for E2

\[ K_{\text{m}} \] is suggested by its function as a DNA repair enzyme (23). While E2

\[ k_{\text{cat}} \] appears to have a unique function in (cytoplasmic) ubiquitin-dependent proteolysis (21), the possibility of an additional role in nuclear ubiquitin metabolism, either as a conjugating enzyme, or possibly as a protein that functions to carry activated ubiquitin derived from E1-ubiquitin into the nucleus, cannot be excluded at present.

Acknowledgments—We are grateful to Sei-ichi Matsui for a generous gift of crude calf thymus histones and for advice in histone purification, and to Lynne Graziani for technical assistance in some of the experiments.

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