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Affinity Chromatography Using Protein Immobilized via Arginine Residues: Purification of Ubiquitin Carboxyl-Terminal Hydrolases†

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Received February 27, 1989; Revised Manuscript Received June 8, 1989

ABSTRACT: 4-(Oxoacetyl)phenoxyacetic acid (OAPA) forms a stable, covalent bond between its glyoxal group and the guanidino group of arginine and arginine derivatives [Duerksen, P. J., & Wilkinson, K. D. (1987) *Anal. Biochem. 160*, 444–454]. Studies were carried out to determine the chemical nature of this linkage, and the structure of the stable adduct between OAPA and methylguanidine was elucidated. The stable product results from an internal oxidation–reduction of the Schiff base adduct to form a cyclic α-aminoamide, 4-[4-(carboxymethoxy)phenyl]-2-(methylimino)-5-oxoimidazolidine. OAPA coupled to polyacrylamide beads was used to immobilize ubiquitin via its arginine residues, and the resulting affinity support was shown to specifically and reversibly bind a previously described enzyme, ubiquitin carboxyl-terminal hydrolase [Pickart, C. M., & Rose, I. A. (1985) *J. Biol. Chem. 260*, 7903–7910]. The resin was then used to isolate three newly identified ubiquitin carboxyl-terminal hydrolytic activities, which did not bind to ubiquitin immobilized via lysine residues. Significant purification was achieved in each case, and one isozyme was further purified to homogeneity.

One of the most useful tools in modern protein purification has been affinity chromatography, in which the biological ligand-binding properties of a desired protein or group of proteins are exploited to separate it from other macromolecules. The most frequently used methods of preparing affinity resins focus on covalent attachment of the protein to the bead via nucleophilic side chains, such as those of lysine or cysteine residues [reviewed by Srere and Uyeda (1976)]. Useful as such resins are, they are limited in application, in large part because a number of alternative sites of attachment are available, which results in many possible binding configurations. Even when the number of possible linkages are small, the location of the reactive residue will determine what portion of the ligand is exposed to the media. Frequently, a protein of interest will be unable to recognize and bind to the portion of the ligand exposed.

We wished to develop the chemistry to present alternative regions or conformations of ligands coupled to solid matrices for use as affinity supports. Immobilization via arginine residues would be a logical alternative to currently available supports since there are often fewer of these residues than lysine residues in proteins. We have previously synthesized and characterized a linker molecule, 4-(oxoacetyl)phenoxyacetic acid (OAPA),[1] which was capable of binding to arginine residues of a ligand protein via a glyoxal functional group, and also of being linked to a solid matrix via a carboxyl group. Our studies indicated that many proteins immobilized in this manner were irreversibly bound (Duerksen & Wilkinson, 1987). Since this characteristic is highly desirable for affinity chromatographic work, we sought to chemically define the reaction through X-ray crystallography and nuclear magnetic

† This work was supported by grants awarded to K.D.W. from the NIH (GM30308) and the NSF (DMB-8703828). K.D.W. is the recipient of an NIH Research Career Development Award (HL01929).
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[1] Abbreviations: OAPA, 4-(oxoacetyl)phenoxyacetic acid; UCH, ubiquitin carboxyl-terminal hydrolase; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography, Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Ub-OAPA-R, the affinity resin obtained by coupling ubiquitin to OAPA immobilized on polyacrylamide beads as described by Duerksen and Wilkinson (1987).

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resonance studies, and these results are reported here.

We also wished to test this method of immobilization for its utility as an affinity support. We have previously shown that OAPA reacts primarily with three closely spaced arginine residues in the protein ubiquitin (Duerksen-Hughes et al., 1987), making it likely that a single face of the ubiquitin molecule would be the site of immobilization. Accordingly, we have used a polyacrylamide support with ubiquitin immobilized via arginine residues to isolate enzymes with putative ubiquitin-binding sites. Our laboratory has recently investigated enzymes that hydrolyze various amide and ester leaving groups from the carboxyl terminus of ubiquitin. We have reported separating four distinct activities from calf thymus and their partial purification (Mayer & Wilkinson, 1989). One of these four similar isozymes appears to be a homologue of a previously described and purified activity (Pickart & Rose, 1985). Of these four activities, only the latter bound to ubiquitin immobilized via lysine residues to activated Sepharose beads, although previous work had indicated that all four activities possess ubiquitin-binding sites. This support reported here is capable of significant purification of each of these enzymes, three of which do not bind to ubiquitin immobilized via lysine residues. Thus, this method of immobilization is useful in presenting alternative portions of the ligand molecules for use as affinity supports.

Materials and Methods

Materials. 4-(Oxoacetyl)phenoxyacetic acid (OAPA) was synthesized as described earlier (Duerksen & Wilkinson, 1987). Methylguanidinium chloride was purchased from Sigma Chemical Co., St. Louis, MO. Ubiquitin was purified from bovine erythrocytes as described previously (Haas & Wilkinson, 1985). Ubiquitin ethyl ester was synthesized by trypsin-catalyzed transpeptidation with glycylglycine ethyl ester (Wilkinson et al., 1986), and ubiquitin was immobilized via its arginyl residues as described earlier (Duerksen & Wilkinson, 1987). Calf thymus was obtained from Pel Freeze Biologicals, Rogers, AK, and electrophoresis chemicals were from Bio-Rad Laboratories, Richmond, CA.

Crystal Structure of OAPA. Platelike crystals of OAPA were obtained from evaporation of an aqueous solution. A clear, colorless specimen of OAPA was affixed to the end of a glass capillary and the capillary mounted on a goniometer head of a Syntex (Nicolet) $P2_1$ four-circle diffractometer. Cell dimensions were obtained by least-squares refinement of 25 centered reflections [$4.00° \leq 2\theta \leq 31.79°$; λ(MoKα) = 0.71069 Å] and were a = 3.996 Å, b = 11.152 Å, and c = 24.14 Å. The Wyckoff ω scan method was used with a variable scan rate of 3.91–14.65°/min (scan/background = 1). Intensity measurements of two standards every 198 reflections were made. The average intensity loss of the standards was only 8% (over a period of 14.2 h). Intensities (2θ = 3–55°) were measured for 1538 reflections of which 1324 unique reflections displayed $F \geq 2.5\sigma(F_0)$. The structure was solved by direct methods on a Data General Eclipse S/140 computer with Nicolet SHELXTL software. The space group was uniquely determined to be orthorhombic, $P2_12_12_1$ (No. 19) with systematic absences: hkl; $h00$, $h = 2n + 1$; $0k0$, $k = 2n + 1$; and $00l$, $l = 2n + 1$. All nonhydrogen atoms were refined anisotropically. All hydrogen positions were located from electron density maps and allowed to refine freely until the final stages of the refinement, when they were fixed. The final electron density difference map was featureless with the maximum positive peak of 0.175 e/Å^3 at a distance of 0.93 Å from a hydrogen-bonded water molecule. Blocked-cascade least-squares refinement of 154 variables gave a value of R = 0.0392 (where $R = \sum||F_0| - |F_c||/\sum|F_0|$). On the last cycle of the least-squares refinement the ratio of the maximum shift to the estimated standard deviation was 0.015. Correction was made for the small intensity decay over the data collection period. Adsorption correction was by semiempirical (ψ-scan) methods. A weighting scheme utilizing weights of the form $w = [\sigma^2(F) + |g|F^2]^{-1}$ (where $g = 2 \times 10^{-4}$) was applied. Scattering factors were those used for neutral atoms (Cromer & Waber, 1974).

Preparation of the OAPA–Methylguanidine Adduct. For preparative synthesis, OAPA (500 mg) and methylguanidine (310 mg) were incubated in 10 mL of water or deuterium oxide containing potassium carbonate (1 g). After reacting for 16 h at 37 °C, 20 volumes of ethanol were added to precipitate the potassium carbonate. After filtration, the ethanol solution was added to twice its volume of diethyl ether to precipitate the product. The product was dried overnight in vacuo and dissolved in deuterium oxide for NMR studies.

Characterization of the OAPA–Methylguanidine Adduct. NMR[1] data were collected with either a Nicolet 360-MHz instrument (for proton spectra) or a 200-MHz instrument (for ^{13}C spectra), using dimethyl-d_6 sulfoxide or deuterium oxide as the solvent and tetramethylsilane as the reference. HPLC[1] was accomplished by using a C-18 reverse-phase column (Alltech Associates Inc., Deerfield, IL) with a mobile phase of 0.01% trifluoroacetic acid in 15% acetonitrile. Absorbance spectra were recorded on a Aminco DW2-C spectrophotometer.

Preparation of the Ubiquitin Affinity Column. The ubiquitin affinity resin (Ub-OAPA-R)[1] was prepared as described previously (Duerksen & Wilkinson, 1987). To block the unreacted OAPA sites, the beads were treated with sodium borohydride. After equilibration of the beads in 0.1 M potassium phosphate, pH 8.0, a 20-fold molar excess sodium borohydride (calculated on the basis of the original OAPA substitution) was added and the mixture was incubated 1 h at room temperature. The pH of the mixture was brought to 3.0 with 1 M HCl in order to discharge the remaining reductant, and the mixture was incubated an additional 30 min. The resin was then washed extensively with 50 mM Tris·HCl,[1] pH 7.6. All remaining 2,4-dinitrophenylhydrazine reactivity was eliminated by this treatment. For storage, 0.02% sodium azide was included in the storage buffer (50 mM Tris·HCl, pH 7.6) and the resin was kept at 4 °C. The final concentration of bound ubiquitin was 9.6 mg/mL of resin.

Reversible Binding of Ubiquitin Carboxyl-Terminal Hydrolase. Ubiquitin carboxyl-terminal hydrolase (UCH)[1] was isolated as previously described (Pickart & Rose, 1985). A 0.3-mL ubiquitin affinity column (prepared as described above, except that the ubiquitin substitution was 8.9 mg/packed mL and excess OAPA sites were not blocked) was equilibrated in buffer A (50 mM Tris·HCl, pH 7.6, 1 mM dithiothreitol, 0.5 mg/mL ovalbumin). UCH (20 μL, 0.18 unit/mL) was applied to the column, and the flow was stopped for 1 h to allow binding. After washing with 20–30 column volumes of buffer A, the enzyme was eluted from the column by one of two methods. In one case, a salt gradient from buffer A to buffer B (50 mM Tris·HCl, pH 7.6, 1 mM dithiothreitol, 0.5 mg/mL ovalbumin, 0.5 M sodium chloride) eluted the enzyme, with the peak of activity eluting at 53 mM sodium chloride. Alternatively, a 10-fold excess of free ubiquitin (30 mg in 300 μL of buffer A) was applied, the flow was halted for 20 min, and then the column was washed with several column volumes of buffer A. Subsequently, a second 8-fold excess of free ubiquitin was applied, the flow halted, and the column washed as before. Ubiquitin-containing fractions (detected by ab-

sorbance at 280 nm) resulting from each application were pooled and applied to a 1-mL DE-52 column equilibrated in buffer A. After washing with 10–20 column volumes of buffer A to remove free ubiquitin, the retained UCH was eluted with buffer B.

Affinity Purification of Bovine Thymus UCH Isozymes L-1, L-2, and H-2. The ubiquitin affinity column (0.7 mL, 9.6 mg of ubiquitin/packed mL, excess OAPA sites blocked) was equilibrated with equilibration buffer (50 mM Tris·HCl, pH 7.6, 1 mM dithiothreitol) at room temperature. Thymus UCH isozymes were prepared as described previously (Mayer & Wilkinson, 1989). Each enzyme preparation was diluted to a protein concentration of 0.5 mg/mL with equilibration buffer and applied to the column. To saturate the binding capacity of the column, enzyme was applied continuously until the effluent displayed the same level of esterase activity as did the solution being applied. The column was then washed with equilibration buffer, and the activity was eluted by elution buffer (equilibration buffer plus 0.3 M sodium chloride). The fractions containing enzyme activity were pooled and concentrated by ultrafiltration (Amicon, YM 5).

Gel Filtration Purification of Isozyme L-1. Since SDS–PAGE[1] analysis indicated that the greatest purification from the affinity step had been achieved with isozyme L-1, this enzyme was further purified by gel filtration using one of two methods. A Superose 12A FPLC column (Pharmacia) was equilibrated in 30 mM potassium phosphate, pH 7.9, and a 100-μL aliquot of the affinity-purified L-1 (approximately 3 mg/mL, 0.5 unit/mL) was applied. The flow rate was 0.4 mL/min, and the absorbance at 280 nm was monitored. Alternatively, the concentrated enzyme was applied to a Bio-Gel P-60 column (200 mL) equilibrated in 50 mM Tris·HCl, pH 7.6, and 1 mM dithiothreitol and eluted with the same buffer. Fractions were collected, and the activity was located as described below.

Assay of Esterase Activities. Appropriate dilutions of the enzyme solution were incubated with 10 mM Tris·HCl, pH 8.0, 10 mM dithiothreitol, and 0.1 mg/mL ubiquitin ethyl ester for 10 min at 37 °C. For assays of the latter stages of purification, 1 mg/mL ovalbumin was included in the incubation to prevent losses due to adsorption of the purified protein. The sample (20 μL) was applied to a HPLC C-8 column and the elution of products monitored at 205 nm. Conversion of ester to ubiquitin was measured and the activity calculated as previously described (Wilkinson et al., 1986). Activities are reported in terms of international units (micromoles of ester hydrolyzed to ubiquitin per minute).

Other Methods. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by Coomassie and/or silver staining (Bio-Rad kit), was used to monitor enzyme purification. Protein concentrations were determined by the modified Bradford method (Bradford, 1976; Bearden, 1978), using bovine γ-globulin as the standard.

Results

Model Studies on the Reactivity and Structure of Guanidino–OAPA Adducts. (1) Crystal Structure of OAPA. In order to define the three-dimensional structure of OAPA and to determine the hydration state of the two carbonyl carbons, we determined the crystal structure of OAPA. Figure 1 shows a labeled drawing of the molecule. As shown, OAPA crystallized as a planar, monohydrated *gem*-diol (formed by the addition of a molecule of water to the aldehydic functionality). Intramolecular bond distances and angles were unexceptional. The packing diagrams (not shown) illustrate extensive hydrogen bonding throughout the crystal structure, with OAPA forming rows of hydrogen bonds in a head to tail fashion. Table I gives the atom coordinates of OAPA.

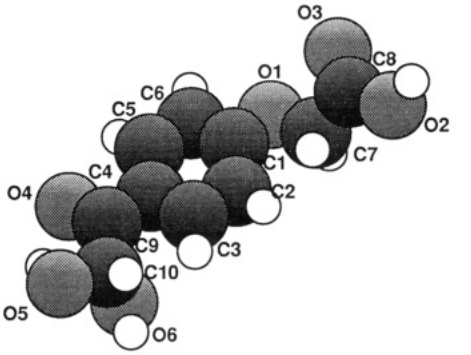

FIGURE 1: Structure of OAPA as determined by X-ray crystallography. Crystals were grown in water and analyzed as described under Materials and Methods. Atom coordinates are given in Table I.

Table I: Atom Coordinates of OAPA (Å)

	x	*y*	*z*		*x*	*y*	*z*
C-1	0.289	1.382	0.179	O-4	−1.732	−3.166	0.282
C-2	1.330	0.492	−0.025	O-5	−0.060	−4.873	1.305
C-3	1.049	−0.854	0.165	O-6	1.177	−3.997	−0.462
C-4	−0.260	−1.315	0.170	H-C2	2.250	0.716	−0.073
C-5	−1.297	−0.395	−0.012	H-C3	1.784	−1.517	0.272
C-6	−1.031	−0.943	−0.174	H-C5	−2.226	−0.780	−0.047
C-7	1.757	3.259	−0.263	H-C6	−1.741	1.602	−0.254
C-8	1.643	4.747	−0.089	H-C7a	2.249	2.868	0.501
C-9	−0.589	−2.730	0.386	H-C7b	1.106	−3.387	1.400
C-10	0.526	−3.727	0.732	H-O2	2.761	6.204	0.021
O-1	0.441	2.734	−0.339	H-O5	−0.850	−4.904	1.010
O-2	2.831	5.316	−0.125	H-O6	1.987	−4.423	−0.236
O-3	0.613	5.32	0.081				

(2) Reaction of OAPA with Methylguanidinium Chloride. In order to elucidate the type of stable linkages formed upon immobilization of proteins on the OAPA affinity support, we have investigated the structure of the adduct between OAPA and methylguanidine.

When OAPA (15 mM) and methylguanidine (250 mM) are mixed at pH 10.6 and 37 °C, 95% of the OAPA is consumed in less than 1 min, as measured by absorbance spectroscopy, NMR, and HPLC. The absorbance at 253 nm initially decreases and then rises back to slightly above the original level with a half-time of 6 min, and a similar absorbance change is observed when phenylglyoxal is used as the arginine reagent. These spectral changes probably reflect the formation of an initial aminol adduct, followed by dehydration to the Schiff base.

When the reaction mixture is examined using HPLC, monitored at 253 nm, two intermediates are observed eluting at 12 and 13 min. With time (~2 h), these peaks diminish and peaks appear eluting at 4, 6.5, and 10 min. The 4- and 6.5-min peaks are also formed if OAPA is treated with base or with catalytic amounts of methylguanidinium chloride, suggesting that they have arisen from a Cannizaro-like rearrangement of OAPA. The peak at 10 min forms only in the presence of stoichiometric amounts of methylguanidine and accounts for 70% of the reaction product as determined by integration of the area of the peaks observed at 253 nm. This suggests that the latter peak is the major product formed under these conditions. This compound is stable to dilution and the acidic chromatography buffer, since, upon rechromatography of this peak, only the 10-min peak is observed.

During the course of the reaction between OAPA and methylguanidine in deuterium oxide, ^{1}H NMR spectra were taken at several time points. These spectra indicate that all free OAPA is consumed rapidly upon mixing and that a

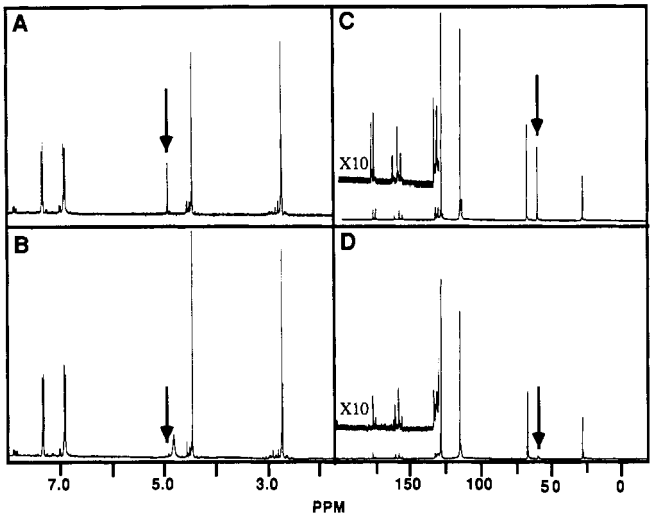

FIGURE 2: Proton NMR spectra of the OAPA–methylguanidine adduct formed in water (panel A) and in deuterium oxide (panel B). The water peak was suppressed in the spectrum of the water-formed compound, but not in the case of the deuterium oxide formed compound. ^{13}C NMR spectra of the OAPA–methylguanidine adduct formed in water (panel C) and deuterium oxide (panel D). The arrows indicate resonances that are absent when the adduct is formed in deuterium oxide.

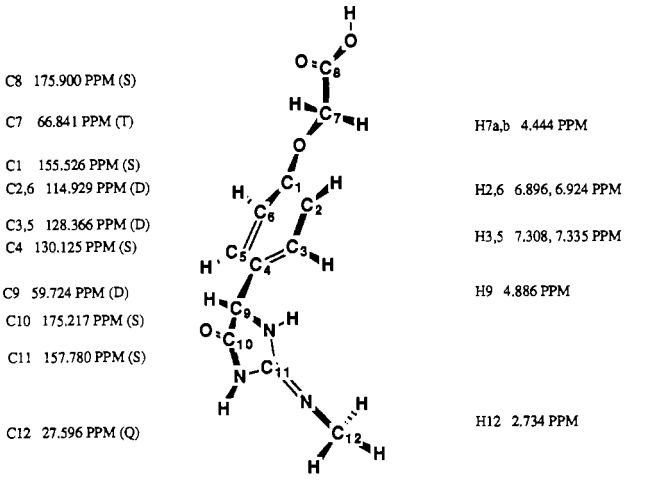

FIGURE 3: Proposed structure and assignment of the NMR resonances of the OAPA–methylguanidine adduct. The structure shows the numbering system used. Chemical shifts of the ^{13}C resonances are reported under proton-decoupled conditions, and the proton coupling patterns are indicated in parenthesis following the chemical shifts.

complex has formed that displays multiple aromatic resonances. As the reaction proceeds, the aromatic resonances coalesce into more clearly defined peaks, somewhat upfield of the original OAPA resonances with a time course consistent with the formation of the 10-min peak observed upon HPLC. Concurrently, the methyl resonance (from the methylguanidine) rapidly shifts downfield approximately 0.5 ppm upon complex formation and, within several hours, moves back toward the original position.

(3) Structure of the OAPA–Methylguanidine Adduct. A proton NMR spectrum of the isolated product formed under these conditions in water is shown in Figure 2A. When the reaction is run in deuterium oxide, the peak at 4.780 ppm (~1 proton) is not present, demonstrating that this proton is solvent derived (Figure 2A,B, arrows).

^{13}C NMR spectra of the two samples were also analyzed. Shown are the proton-decoupled ^{13}C spectra from the compounds formed in water (Figure 2C) and in deuterium oxide (Figure 2D). Clearly, a resonance at 59.7 ppm is present in the compound formed in water but is broadened or greatly reduced in intensity in the compound formed in deuterium oxide (Figure 2C,D, arrows). The proton and carbon spectra are consistent with the formation of an additional methylene carbon upon adduct formation and indicate that this carbon bears a solvent-derived proton. When the solvent-derived atom is deuterium, the relaxation time of the carbon is lengthened and results in a diminished intensity for that carbon. Additionally, resonances at 175.2 and 130.1 ppm are somewhat diminished in intensity when the product is formed in deuterium oxide.

To account for these results, the structure shown in Figure 3 is proposed. This structure arises from an internal oxidation–reduction of the 1/1 adduct to form an α-amino amide, 4-[4-(carboxymethoxy)phenyl]-2-(methylimino)-5-oxoimidazolidine. The ^{1}H and ^{13}C NMR spectral assignments, both proton coupled and decoupled, are also given in Figure 3 and are consistent with this structure. Both a solvent-derived proton and the carbon bonded to that proton are clearly identified. It should also be noted that the NMR data indicate that this carbon is not bonded to any other protons, as predicted from the proposed structure. The remaining assignments are also reasonable and are based on published values for similar environments. This structure is analogous to that determined by Soman et al. (1985) for the adduct of (*p*-nitrophenyl)glyoxal and canavanine and explains the stability of the linkage previously observed (Duerksen & Wilkinson, 1987).

Characterization of an Affinity Support (Ub-OAPA-R) Formed by Attaching Ubiquitin to OAPA-Derivatized Polyacrylamide Beads. (1) Biospecific Binding of Ubiquitin Carboxyl-Terminal Hydrolase. To assess if proteins with a known ubiquitin-binding site could be adsorbed by the Ub-OAPA-R column, we tested the binding of ubiquitin carboxyl-terminal hydrolase (UCH) to this support. This enzyme bound to the Ub-OAPA-R column without detectable activity eluting in the flow-through. It was quantitatively eluted from the immobilized ubiquitin with either salt or free ubiquitin (two applications in 10-fold excess over the amount of immobilized ubiquitin, data not shown). UCH also binds to ubiquitin–Sepharose (Pickart & Rose, 1985), and this characteristic provides a major step in the purification of the enzyme. The similar binding behavior of UCH to the Ub-OAPA-R column demonstrates the ability of this latter resin to serve as an affinity support for the purification of at least one ubiquitin-binding protein.

The fact that UCH elutes from the column at a relatively low sodium chloride concentration (53 mM) suggests that the binding is not extremely tight. The binding, however, does appear to be quite specific, as a 10-fold excess of free ubiquitin was able to elute the enzyme, while the presence of 0.5 mg/mL ovalbumin present in all buffers did not interfere with the UCH binding, nor cause its elution from the column.

(2) Blocking of Excess OAPA Sites. The presence of Tris buffer (an inhibitor of arginine binding) during protein application and elution should prevent the applied protein from binding to excess OAPA sites which exist after coupling of the ligand (Duerksen & Wilkinson, 1987). Nevertheless, in order to eliminate the possibility that protein in crude preparations could bind to these excess OAPA sites, they were reduced by treatment with sodium borohydride after the ligand protein, ubiquitin, had been bound. This reduction appears to quantitatively block OAPA sites, as the beads lost their (2,4-dinitrophenyl)hydrazine reactivity. It did not appear to affect the immobilized ubiquitin in any significant way, as the

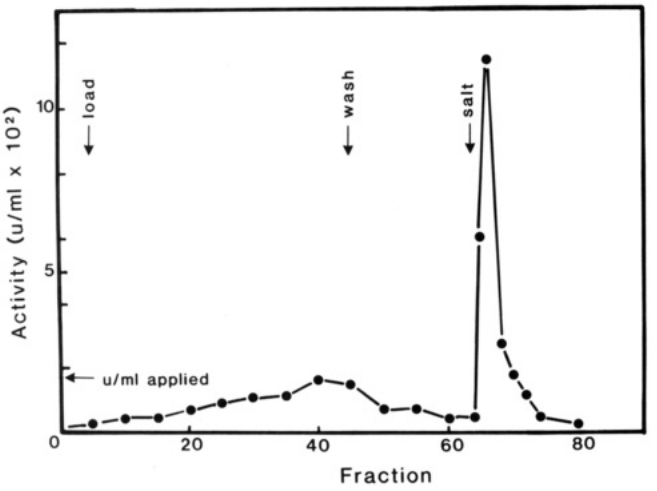

FIGURE 4: Salt elution of UCH isozyme L-1 from affinity resin. Enzyme (0.019 unit/mL) was applied to the ubiquitin affinity column until the effluent contained the same level of activity as the sample applied. After saturation of the binding capacity was achieved, the column was washed with equilibration buffer and eluted with the application of 0.3 M NaCl as described under Materials and Methods. Activities of fractions were determined by using ubiquitin ethyl ester as a substrate.

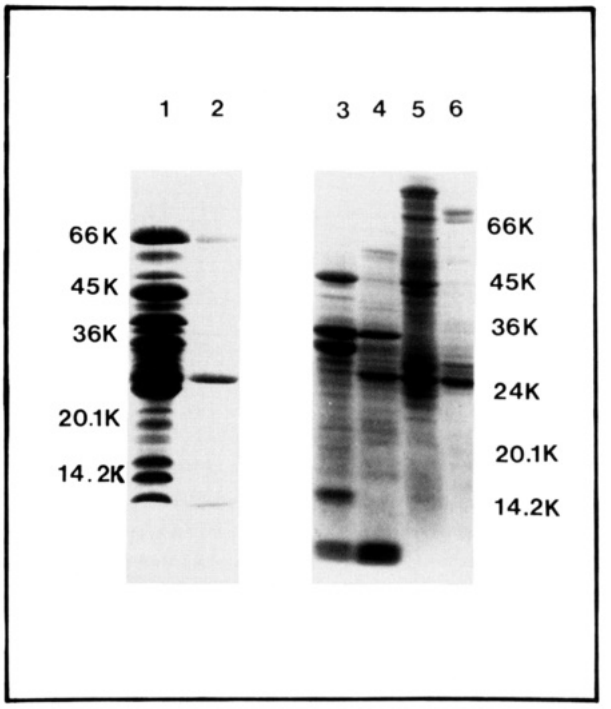

FIGURE 5: Affinity purification of UCH isozymes L-1, L-2, and H-2. Lanes 1 and 2 contain 0.056 esterase unit of isozyme L1, lanes 3 and 4 contain 0.008 esterase unit of isozyme L-2, and lanes 5 and 6 contain 0.003 esterase unit of isozyme H-2. Lanes 1, 3, and 5 show the preparations before the affinity step; lanes 2, 4, and 6 show the preparations after the ubiquitin affinity step.

behavior of the column in reversibly binding UCH isozymes was identical before and after the borohydride treatment (data not shown).

(*3*) *Reversible Binding of Thymus UCH Isozymes.* Thymus contains three UCH isozymes (in addition to the reticulocyte homologue) which can be resolved from each other (Mayer & Wilkinson, 1989). We report here that each of these three enzymes binds reversibly to the Ub-OAPA-R column. Figure 4 shows an activity profile obtained upon binding and salt elution of UCH isozyme L-1. As expected, when large amounts of the enzyme are applied, the column becomes saturated, and increasing amounts of activity are unabsorbed. When smaller quantities (i.e., 2×10^{-3} unit) were loaded onto the OAPA–ubiquitin column, the binding was essentially quantitative, as less than 5% of the activity eluted in the void volume. The corresponding profiles for isozymes L-2 and H-2 were found to be similar. In these experiments, a 0.3-mL column exhibited a binding capacity of 0.31 unit of isozyme L-1, 0.58 unit of isozyme H-2, and 0.38 unit of isozyme L-2. In all cases, the recovery of activity upon affinity chromatography was between 50 and 95%.

(*4*) *Analysis of Affinity-Purified UCH Isozymes.* Although 0.1 M NaCl was a sufficient salt concentration to elute isozyme L-2 and isozyme H-2 from the affinity resin, 0.2 M NaCl was necessary to completely elute the bound isozyme L-1. This would suggest that isozyme L-1 is bound more tightly than the other two enzymes. To ensure that all bound enzyme was eluted from the column, it was eluted with buffer containing 0.3 M NaCl in all three cases.

Significant purification by affinity chromatography was achieved in each case. Figure 5 shows an SDS–PAGE separation of the proteins present in the various steps of purification. Lanes 1 and 2 contained 0.056 unit of isozyme L-1, lanes 3 and 4 contained 0.008 unit of isozyme L-2, and lanes 5 and 6 contained 0.003 esterase unit of isozyme H-2. The odd-numbered lanes show the samples before affinity purification, and the even-numbered lanes show the preparations after affinity purification. The affinity-purified preparation of isozyme H-2 contains primarily polypeptides with apparent molecular weights of 68 000, 64 000, 30 000, and 25 000. The affinity-purified preparation of L-2 is enriched in at least three bands, with apparent molecular weights of 35 000, 29 000, and approximately 10 000. Finally, the affinity-purified preparations of L-1 showed apparent molecular weights of 66 000, 25 000, and approximately 10 000.

(*5*) *Further Purification of Isozyme L-1.* The purification obtained upon affinity chromatography of partially purified isozyme L-1 (Mayer & Wilkinson, 1989) is striking (Figure 5, lanes 1 and 2) with a large enrichment of only three proteins. The recovery of activity upon affinity chromatography has ranged from 50 to 100% in different preparations. Because of the purity of these preparations, we further purified this isozyme. Gel filtration of this sample (P-60 column) yielded a single peak of activity copurifying with the 25 000-dalton polypeptide. Analysis of the active fractions from gel filtration by SDS–PAGE shows that this step removes both the high and low molecular weight contaminants evident in Figure 5 (data not shown). The final gel filtration step results in another 2-fold purification and approximately 30% recovery of activity. The loss of enzyme activity is probably due to oxidation or denaturation of the active enzyme. Experiments have shown that both heat- and oxidation-induced damage, as well as repeated cycles of freezing and thawing, contributes to the loss of enzymatic activity (see below). Further optimization of the purification procedure is currently in progress in our laboratory.

(*6*) *Characterization of Isozyme L-1.* Further characterization studies were carried out on the purified L-1. A molecular weight of 25 000 ± 500 was estimated by both SDS–PAGE analysis and gel filtration. Consistent with earlier reported observations on the crude enzyme (Mayer & Wilkinson, 1989), the purified enzyme is inactivated in the absence of thiol reagents such as DTT but recovers some activity when incubated in the presence of such thiol reagents. A loss of activity is also noted when the purified enzyme is incubated at 25 °C or repeatedly frozen and thawed. Inclusion of 1 mg/mL ovalbumin in the assay mixture partially protected

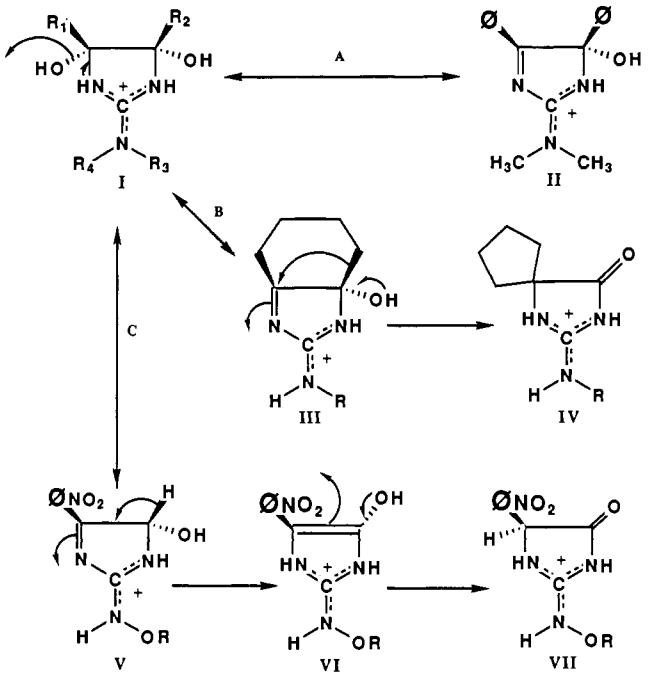

FIGURE 6: Rearrangement of carbinolamine adducts. Structure I is the general structure of the initial adduct formed upon condensation of guanidino compounds with dicarbonyl reagents. In pathway A the adduct of benzil and (1,1-dimethylamino)guanidine undergoes dehydration to give II. In pathway B the adduct of 1,2-cyclohexanedione and arginine first undergoes dehydration followed by ring contraction to give IV. In pathway C the adduct of (*p*-nitrophenyl)glyoxal and canavanine undergoes dehydration followed by a Cannizzaro-like rearrangement to give VII.

against this loss of activity. Comparison of the absorbance at 280 nm with the protein concentration as determined by the modified method of Bradford (Bearden, 1978) indicates that the protein has a very low absorbance at 280 nm. An absorbance spectrum of the purified protein showed that the absorbance maximum occurs at approximately 275 nm, with the absorbance at 280 approximately 0.14 for a 1 mg/mL solution. This suggests that the protein is low in tryptophan or reacts abnormally in the protein assays used. L-1 is a somewhat acidic protein, as it was retained on the DE-52 column at pH 7.5 during the early stages of purification. The N-terminus of the molecule appears to be blocked, and sequence analysis of selected tryptic fragments is currently in progress.

Discussion

Structure of the Linkage Formed When Proteins Are Immobilized via Arginine Residues. A number of studies have focused on the chemistry of the reactions of dicarbonyl reagents with guanidino compounds (Takahashi, 1968; Patthy & Thesz, 1980; Soman et al., 1985). Many more studies have exploited these arginine-specific reagents to obtain information concerning relationships between protein structure and function in a large number of proteins [Lundblad and Noyes (1984) and references cited therein]. Although a variety of products that result from reactions between these arginine reagents and the guanidino functionality have been described, there are some common themes. In each case, the initially formed adduct appears to be a carbinolamine (I), as shown in Figure 6. At this point, the adduct has a number of possible fates. It can dissociate, yielding the original reagents. Alternatively, it can be stabilized either by the addition of a second molecule of the arginine reagent (Takahashi, 1968, 1977) or by the presence of borate (Patthy & Smith, 1975; Riordan, 1973). A third possibility which is germane to the present work is the stabilization by rearrangement of the initial adduct (Figure 6). A number of factors have been suggested to be relevant in determining the characteristics of these guanidino–dicarbonyl adducts, including the pK_a of the guanidino group (Patthy & Thesz, 1980), the equilibrium between the amino and imino forms of the arginine derivative, and the electron-donating or -withdrawing groups present on the reactants (Soman et al., 1985).

All of the rearrangements shown in Figure 6 have in common an initial dehydration of the carbinolamine to the Schiff base. When Nishimura and Kitajima (1979) reacted benzil with (1,1-dimethylamino)guanidine, the product they obtained was the hydroxyimidazole derivative of benzil (II), as shown resulting from path A, Figure 6. In this case, the conjugation of electrons into the phenyl rings stabilizes the adduct. A second type of product was first observed by Toi et al. (1967), when they reacted 1,2-cyclohexanedione with arginine. Path B, Figure 6, shows that, in this case, labilization of the hydroxyl proton results in a ring contraction forming N^5-(4-oxo-1,3-diazaspiro[4,4]non-2-ylidene)-L-ornithine (IV). Finally, Soman et al. (1985) obtained the 5-(*p*-nitrophenyl)-4-oxoimidazolidine derivative of canaliline (VII) after reacting canavanine with (*p*-nitrophenyl)glyoxal. This product results from abstraction of the proton α to the Schiff base followed by a Cannizzaro-like rearrangement, resulting in an internal oxidation–reduction. The results reported here indicate that OAPA reacts with methylguanidine in a manner quite similar to this latter process, as the analagous product was obtained.

It should be emphasized at this point that the means by which this stable product was obtained in these two examples differs significantly. In the case of Soman and his co-workers, the nonphysiological molecule canavanine was chosen as the guanidino reagent rather than arginine. Stabilization of the adduct was attributed to the electronic properties of the arginine derivative. Thus, this reaction is clearly not applicable to arginine residues in proteins. In our case, the stable adduct accumulates because of the electronic properties of the reagent rather than a modified arginine. Thus, the chemistry can be applied directly to the arginine residues present in biologically relevant proteins and peptides, allowing stable complexes to be formed and studied.

Our results presented here outline a number of points. First, the crystal structure of OAPA is straightforward and confirms the structure reported in previous studies. The aldehydic carbonyl is hydrated, and extensive hydrogen bonding is apparent. The extended conformation of this reagent would furnish a spacer arm of approximately 10 Å between the carboxylic acid attachment site and the keto aldehyde which would react with the arginine residues in proteins. Thus, reversible interactions of the immobilized reagent and proteins or peptides should be readily attained. The unique characteristic that makes OAPA particularly attractive as an arginine reagent and as an affinity chromatographic linker is the exceptional stability of the adduct after the irreversible rearrangement.

Purification of Ubiquitin Carboxyl-Terminal Hydrolases. The novel linkage described here, yielding ubiquitin immobilized via its arginine residues, has been shown to result in an affinity support that reversibly and specifically binds at least four distinct enzymatic activities involving ubiquitin-binding sites. In contrast, the previously described ubiquitin–Sepharose column (Ciechanover et al., 1982) is capable of interacting as a bioaffinity support for only one of those four enzymes, UCH (Mayer & Wilkinson, 1989). With the Ub-OAPA-R column, UCH is bound and can be released upon the appli-

cation of either salt or free ubiquitin. This behavior is that expected for a bona fide affinity column, and these results encouraged us to apply this affinity column to the purification of three newly identified enzymes from crude preparations.

The fact that enzymes L-1, L-2, and H-2 all bound to the Ub-OAPA-R column, while exhibiting no detectable binding to the ubiquitin–Sepharose column, substantiates our initial premise, that a new and different population of ubiquitin-binding molecules is amenable to purification and study using this type of immobilized ubiquitin. Ubiquitin affinity resin prepared in this manner is expected to expose a population of ubiquitin molecules to the mobile phase in a unique and relatively homogeneous orientation. Our studies reported earlier indicate that the primary sites of reactions between free OAPA and ubiquitin are at arginines 42, 72, and 74, all within a few angstroms of each other (Duerksen-Hughes et al., 1987). Hence, one would expect that the majority of immobilized ubiquitin molecules are bound on that face of the molecule, exposing the other faces and domains of ubiquitin for biospecific interactions. Potentially, the same alteration of specificity could be obtained with other systems when this type of immobilization is applied. Clearly, this could be of great advantage in the study of systems amenable to this technology.

Although only the purification of L-1 is quantitated here, significant progress in the purification of both L-2 and H-2 is achieved by the use of this column. This affinity chromatographic step will therefore contribute significantly to the purification of L-2 and H-2. It is difficult to further describe the binding specificity of this column since we have no information on whether any of the proteins bound to the present column are also bound by the ubiquitin–Sepharose column previously used.

Another point worthy of mention concerns the other proteins that bound to the affinity column along with the UCH isozymes. As can be seen by the gel in Figure 5, the binding of proteins to the column appeared to be quite limited, with only marginal amounts of nonspecific lysate proteins being retained during the loading and washing of the column. Ubiquitin is implicated in a large number of protein–protein interactions in addition to those involved in protein degradation. Some of these ubiquitin-interacting proteins have been described, the existence of others has been suggested, and still others may exist. It is tempting, therefore, to speculate that these proteins, which copurify with L-1 on the affinity resin, may fall into one of these catagories of ubiquitin-binding proteins. Clearly, much work remains to be done to investigate these intriguing possibilities.

The elucidation of the pathways involved in ubiquitin conjugation and deconjugation with various proteins will be a major advance in the understanding of ubiquitin's role in the control of cellular homeostasis and will very likely also explain some aspects of cell to cell interactions. For these studies, homogeneous preparations of the various proteins involved will be essential. Reported here is the purification to homogeneity of one newly identified member of this family, as well as the partial purification of two others. The availability of these purified proteins is a major contribution to the study of these ubiquitin-associated pathways.

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