Comparative Properties of Genetically Defined Peptidases in Maize†

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ABSTRACT: Four aminopeptidase isozymes (AMP1–AMP4) and an endopeptidase (ENP) from maize have been purified by ammonium sulfate fractionation, DEAE-Sephadex ion-exchange chromatography, and Sephadex G-150 gel filtration. Hydroxylapatite chromatography further purified some of the peptidases. Comparisons of molecular weights, substrate specificities, and responses of peptidases to various reagents were made. The aminopeptidases varied in reactivities with the naphthylamide derivatives of amino acids. AMP1 and AMP3 were most active with the arginine and lysine derivatives; AMP2 was most active with the alanine and glycine derivatives and AMP4 was most active with the phenylalanine, tyrosine, leucine, and tryptophan derivatives. Molecular weights as determined by gel filtration on Sephadex G-150 were 92 000, 86 500, 83 000, 61 000, and 67 600 for AMP1, AMP2, AMP3, AMP4, and ENP1, respectively. AMP2 had a molecular weight of 88 000 as estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. AMP2 hydrolyzed the dipeptide derivatives, glycylglycyl-β-naphthylamide and glycylphenylalanyl-β-naphthylamide. Aminopeptidases were strongly inhibited by Zn^{2+}, Cu^{2+}, Hg^{2+}, and *p*-mercuribenzoate. AMP1, AMP2, and AMP3 were inhibited by 1,10-phenanthroline, whereas AMP4 was not. AMP4 closely resembled aminopeptidases purified from barley grains and pea seeds. ENP was inhibited by *p*-mercuribenzoate and tosyllysine chloromethyl ketone.

It has become increasingly evident that proteases and peptidases have regulatory roles in many biological processes (Reich et al., 1975). Assigning a specific role to a particular enzyme is difficult because such metabolic processes as intracellular protein breakdown, abnormal fragment degradation, and peptide transport and hydrolysis are probably the cumulative effects of a number of proteases and peptidases which can have broad and overlapping substrate specificities. In *Salmonella*, for example, only strains deficient for several peptidases grew more slowly on minimal media than wild-type cultures (Miller & Mackinnon, 1974). Strains lacking only one peptidase grew at normal rates.

We have been examining the genetic control, developmental expression, and biochemical properties of some peptidases in *Zea mays* in an effort to elucidate possible physiological functions of the enzymes. Variations in the electrophoretic mobility patterns of maize aminopeptidases (AMP)[1] are under the control of four structural genes—*Amp1*, *Amp2*, *Amp3*, and *Amp4*—each of which has two or more allelic variants[2] (Ott & Scandalios, 1978). Maize endopeptidase (ENP) is the product of a single genetic locus, *Enp1*, which has five known allelic variants[2] (Melville & Scandalios, 1972; Nielsen & Scandalios, 1974). With one exception, the peptidases occur in all of the maize tissues and developmental stages examined (Vodkin & Scandalios, 1979). AMP2 is unique in its developmental profile and is found only in the immature endosperm during kernel maturation (Scandalios, 1965).

The substrates used to detect the maize peptidases are β-naphthylamide (NA) derivatives of the amino acids. They are convenient chromogenic substrates for peptidases and can be used for histochemical localizations and for direct staining of peptidase activities on gels. Naphthylamidases are found in many plant species, often in multiple forms, but have been purified from only a few (Kolehmainen & Mikola, 1971; Elleman, 1974; Schabort et al., 1978). In this report, we present the conditions for preparative separation of all five maize peptidases, and we compare some of their biochemical properties. We monitored both AMP and ENP activities during purification since the two activities are difficult to separate. Comparisons of substrate specificities, however, were primarily focused on the nonallelic AMP isozymes. Data on AMP3 were obtained for either or both of the electrophoretic allelic variants, AMP3-F and AMP3-S.

Materials and Methods

Maize Stocks. Three maize lines were used for the purification studies. The highly inbred strain W64A was obtained commercially (Ohio Foundation Seed). Lines W10 and T21, though not highly inbred, were homozygous for all peptidase genes. Immature ears from field-grown plants were harvested 18–22 days postpollination and were stored at −50 °C. The peptidase genotypes for these three lines are as follows: W64A = *Amp1-S*, *Amp2-S*, *Amp3-S*, *Amp4-S*, and *Enp1-A*; W10 = *Amp1-F*, *Amp2-S*, *Amp3-F*, *Amp4-S*, and *Enp1-0*; T-21 = *Amp1-S*, *Amp2-S*, *Amp3-I*, *Amp4-S*, and *Enp1-B*.

Enzyme Assays and Protein Measurement. Aminopeptidases were assayed at room temperature by using amino acid–β-NA substrates at 0.34 mM in 0.1 M potassium phosphate, pH 7.5, and 0.5% dimethyl sulfoxide unless stated otherwise. The substrates were first dissolved in dimethyl sulfoxide in order to improve solubility. The standard substrates used were Arg-NA for AMP1 and AMP3, Ala-NA for AMP2, and Leu-NA for AMP4. The reaction was stopped by the addition of 2 N HCl after an appropriate incubation

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[1] Abbreviations used: AMP, aminopeptidase; ENP, endopeptidase; NA, β-naphthylamine; BANA, benzoyl-DL-arginine-β-naphthylamide; BAPNA, benzoyl-DL-arginine-*p*-nitroanilide; L*p*NA, leucine-*p*-nitroanilide; HA, hydroxylapatite; DEAE, diethylaminoethyl; CB, column buffer (10 mM potassium phosphate and 1 mM $MgCl_2$, pH 6.5); Na-$DodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate.

[2] The italicized three letter gene symbols, *Amp* and *Enp*, have been used in order to conform with the recommended nomenclature rules for assigning maize gene symbols. *Amp1*, *Amp2*, *Amp3*, and *Amp4* code for electrophoretic variation of the nonallelic isozymes, AMP1, AMP2, AMP3, and AMP4, respectively. F, S, and I are arbitrary designations for allelic variants. For example, *Amp2-S* codes for the isozyme band AMP2-S, which is often denoted simply as 2S. Similarly, endopeptidase variants ENP1-A and ENP1-B (or 1A and 1B in abbreviated form) are allelic products of *Enp1*.

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period. Free naphthylamine was detected at 620 nm after a diazotization reaction (Goldbarg & Rutenberg, 1958). Endopeptidase was assayed similarly by using BANA at 0.26 mM. Peptidases were also assayed with leucine-*p*-nitroanilide (L*p*NA) or with 2-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) by following the release of *p*-nitroaniline at 410 nm (Erlanger, 1961). Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Enzyme Extraction and Protamine Sulfate Treatment. All extraction procedures and other steps were conducted at 4 °C unless otherwise indicated. Immature ears were partially thawed and kernels were removed from the cobs with a knife or a razor blade. Generally 200–500 g fresh weight of immature kernels was used per extraction. For preparations using dry seed instead of immature kernels, 600 g of seed was surface sterilized in 1% NaOCl (1/5 dilution of commercial bleach), rinsed thoroughly, and soaked in distilled H_2O for 24 h at room temperature.

The extraction mixture consisted of 2 mL of extraction buffer/g fresh weight and 0.25 g of polyvinylpolypyrrolidone (PVP, Polyclar-AT)/g fresh weight. The extraction buffer was composed of 50 mM potassium phosphate, 1 mM $MgCl_2$, and 10 mM β-mercaptoethanol, pH 7.5. The tissue was ground in a Waring blender, and the homogenate was squeezed through four layers of cheesecloth to remove excess debris. The homogenate was centrifuged at 9800*g* for 30 min in a Sorvall GSA rotor and the pellet was discarded. Standard centrifugation conditions used throughout the purification procedure were 9800*g* and 30 min. A 2% protamine sulfate solution in CB was added slowly, with stirring, to the crude supernatant in a ratio of 70 mL of protamine sulfate solution/1000 mL of crude supernatant. After 30 min of additional stirring, the mixture was centrifuged and the pellet was discarded.

Ammonium Sulfate Fractionations. The protamine sulfate supernatant was brought to 40% saturation with slow addition of solid ammonium sulfate (Ultrapure, Schwarz/Mann). After 20 min of additional stirring the suspension was centrifuged, and the resulting pellet was discarded. The 40% saturated supernatant was brought to 60% saturation with respect to ammonium sulfate, stirred 30 min, and centrifuged. The resulting supernatant was discarded and the 40–60% ammonium sulfate pellet was redissolved in 20–40 mL of CB. The solution was dialyzed for 16 h against two changes (1000 mL each) of CB containing 0.1 M KCl.

DEAE-Sephadex Ion-Exchange Chromatography. After dialysis the 40–60% ammonium sulfate fraction was clarified by centrifugation and applied, at a rate of 35–40 mL/h, to a DEAE-Sephadex A-50 column (40 × 2.6 cm) equilibrated with CB containing 0.1 M KCl. The column was washed with 400 mL of 0.1 M KCl CB, and a 1200-mL linear KCl gradient generally consisting of 0.1–0.23 M KCl CB was applied to the column. Fractions of 11 mL were collected and were assayed for activities with Arg-NA, Leu-NA, Ala-NA, and BANA as substrates. Isozyme peaks were separately pooled, and the protein was precipitated by adding solid ammonium sulfate to 65% saturation. The precipitated protein was stored at 4 °C until used for further purification procedures. The storage period was generally not more than 1–3 weeks, but good activities are recoverable for some of the isozymes stored up to 1 year as an ammonium sulfate precipitate.

Sephadex G-150 Gel Filtration. The precipitated isozyme preparation from ion-exchange chromatography was centrifuged, and the pellet was redissolved in ~10 mL of CB and dialyzed for 6 h against two changes of CB (800 mL each). After dialysis the sample was centrifuged and the clarified supernatant was applied to a Sephadex G-150 column (90 × 2.6 cm) equilibrated with CB. Fractions of 6.8 mL were collected at a rate of 20 mL/h. At this stage in the purification, some of the isozyme activity from the pooled Sephadex G-150 fractions was saved for use in enzyme characterization studies. The isozyme was diluted 1:1 with ethylene glycol and stored at −20 °C. The rest of the preparation was further purified by rechromatography on DEAE-Sephadex, isoelectric focusing, or HA chromatography.

Hydroxylapatite Chromatography. Pooled fractions from Sephadex G-150 sieving were pumped at 35–40 mL/h onto a HA bed surface (Bio-Gel HTP from Bio-Rad) in a 20 × 1.5 cm column equilibrated with CB. After a 250-mL wash with CB, a 600-mL linear phosphate gradient from 0.01 M potassium phosphate and 1 mM $MgCl_2$, pH 6.5, to 0.18 M potassium phosphate and 1 mM $MgCl_2$, pH 6.5, was applied. Fractions of 5.5 mL were collected. Activity regions were pooled and concentrated with vacuum-operated filter units. An equal volume of ethylene glycol was added, and the sample was stored at −20 °C.

Isoelectric Focusing. Isoelectric focusing was conducted with an LKB 8101 (110-mL) focusing column. Duration of the focusing period was generally 44 h at 480–500 V with pH 4.0–6.0 or pH 3.5–5.0 ampholytes. Fractions of 1.1 mL (25 drops) were collected and analyzed. Ampholytes were partially removed from pooled activity fractions by Sephadex G-150 gel filtration.

Electrophoresis. Starch gel electrophoresis using a Tris–citrate, pH 7.0, buffer system and staining for peptidase using NA substrates and Black K salt as the dye coupler were performed as described (Ott & Scandalios, 1978). Staining with peptide substrates using an amino acid oxidase coupled reaction was conducted by the method of Lewis & Harris (1967). Polyacrylamide gel electrophoresis was performed as described by Davis (1964), and the method of Weber & Osborn (1969) was used for $NaDodSO_4$–polyacrylamide gel electrophoresis.

Results

Peptidases, especially AMP2, had higher activities in immature kernels than in dry seed. Most extractions, therefore, were made from immature seed at 18–22 days postpollination. Preparative separation of the isozymes was achieved by DEAE-Sephadex ion-exchange chromatography of a 40–60% ammonium sulfate extract. Figure 1 shows the elution profile obtained for maize lines W64A and W10, each of which possesses a different genotype with respect to the peptidases. In W64A, separation of AMP3-S and ENP1-A was incomplete. Line W10, which possesses a "null" variant (ENP1-0) for endopeptidases, was used to effect removal of endopeptidase activity from aminopeptidases. Endopeptidase activities in this line were ~15% of that in normal lines, and more than half of this residual activity precipitates in the 0–40% ammonium sulfate fraction rather than the 40–60% fraction. Endopeptidase activity substantially separated from aminopeptidases can be obtained by using line T21 which has the ENP1-B variant (data not shown).

There was only a small peak of AMP1 in any of the three maize lines. This was unexpected since the AMP1 isozyme stained intensely on starch gels (Ott & Scandalios, 1978). In order to eliminate the possibility that ammonium sulfate fractionation was preferentially inhibiting AMP1 activity, we applied the protamine sulfate supernatant from one extraction directly to the ion-exchange column without prior concen-

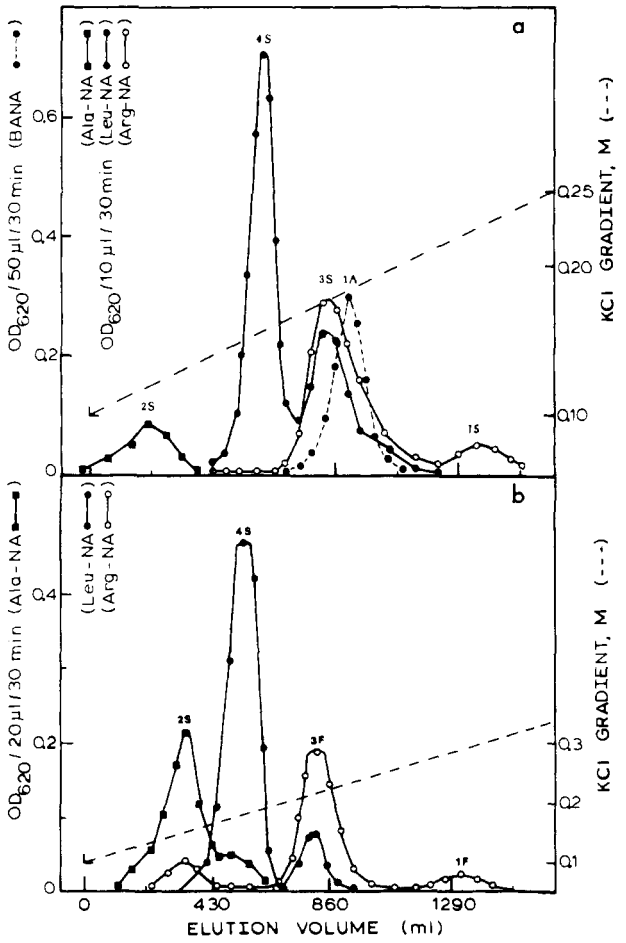

FIGURE 1: Separation of peptidases using DEAE-Sephadex A-50 chromatography. The 40–60% ammonium sulfate fraction was dialyzed and applied to a column as described under Materials and Methods. Fractions were assayed for BANA (●---●), Ala-NA (■—■), Leu-NA (●—●), and Arg-NA (○—○). Isozyme peaks are denoted in abbreviated form by 1S, 2S, 3S, and 4S for aminopeptidase and by 1A for endopeptidase. The maize lines used were (a) W64A, 600 g of dry seed, and (b) W10, 260 g of immature kernels.

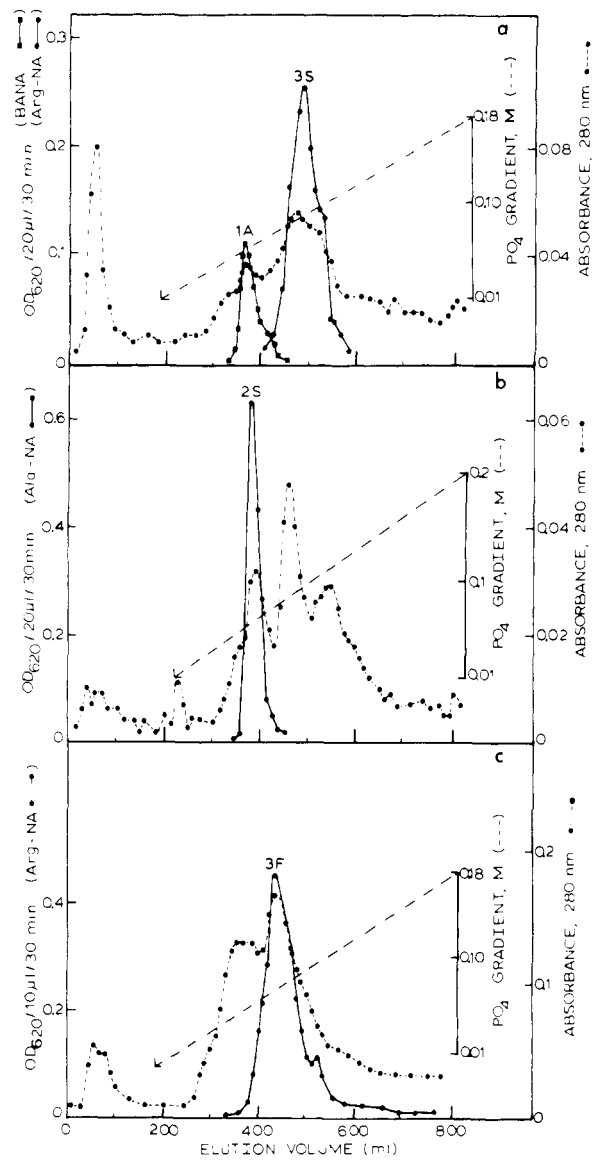

FIGURE 2: HA chromatography of peptidases after gel filtrations. Procedures are given under Materials and Methods. (a) Separation of AMP3-S and ENP1-A; (b) elution of AMP2-S; (c) elution of AMP3-F.

tration by ammonium sulfate precipitation. There is no increased recovery of AMP1 by this method. Either AMP1 was inactivated by some other procedure or it occupies only a small percentage of the total aminopeptidase activity. In contrast, the major aminopeptidase peak from DEAE-Sephadex was always AMP4. However, this isozyme consistently stained less intensely on starch gels than the other aminopeptidases. AMP4 from crude extracts and purified preparation was much less stable to both starch gel and polyacrylamide gel electrophoresis than the other peptidases.

Peptidase isozymes separated by DEAE-Sephadex were each precipitated with ammonium sulfate and were further purified by Sephadex G-150 gel filtration. After this step, preparative separation of all peptidases had been achieved excepting AMP3-S and ENP1-A.

Isoelectric focusing has been extensively investigated as a method for further purification of some of the peptidase isozymes. The isoelectric points of maize peptidases range from pH 4.0 to pH 4.5. Enzyme preparations from Sephadex G-150 were focused by using ampholyte pH ranges 4.0–6.0 or 3.5–5.0 (data not shown). A single sharp activity peak was always obtained for any isozyme column, indicating that no artifactual activity peaks were created by binding of the ampholytes to the enzyme. Recoveries from isoelectric-focusing preparations were generally in the range of 60–70%. However, some isozymes were not stable upon removal of the ampholytes by dialysis against CB or by Sephadex G-150 gel filtration. AMP3 and AMP4 showed large losses (up to 88%) when the ampholytes were separated from the proteins by gel filtration.

AMP3-S and ENP1-A which were not completely separable on DEAE-Sephadex could not be further resolved by isoelectric focusing since their isoelectric pH values are 4.43 and 4.47, respectively. Rechromatography on DEAE-Sephadex with shallower gradients did not improve resolution. Since their molecular weights are in the same range (refer to Figure 3, discussed below), gel filtration techniques did not effect separation. Separation of AMP3-S and ENP1-A was achieved, however, by HA chromatography as shown in Figure 2a. From HA, 63% of ENP1-A activity was recovered. Final purification data are presented in Table I for the endopeptidase variant. Other peptidase isozymes are also purified significantly by elution from HA (Figure 2b,c); 65% of AMP2 activity and 45% of AMP3 activity were recovered. AMP4 did not elute as well from HA as did the other isozymes. Recovery was lower (~25%) and the peak was broad. Quantified purification schedules for each aminopeptidase isozyme (data not shown) were complicated by the fact that the amino-

Table I: Purification of ENP1-A from W64A Mature Seed

fraction	total act. (μmol/h)	total protein (mg)	sp act. [μmol (h mg)$^{-1}$]	% recovery
crude supernatant	488	4790	0.102	100
protamine sulfate supernatant	493	4030	0.122	101
40–60% $(NH_4)_2SO_4$	347	1470	0.236	70
DEAE-Sephadex	151	39.4	3.83	31
Sephadex G-150	125	13.5	9.25	26
hydroxylapatite	78.9	2.64	29.9	16

peptidases had overlapping substrate specificities.

Molecular Weight Determination. Peptidase isozyme preparations purified through the gel filtration step were used for estimates of the native molecular weights on a calibrated Sephadex G-150 column. Samples of 1-mL volume were separately applied to a 1.5 × 50 cm column equilibrated with 9 mM phosphate–120 mM NaCl, pH 7.2, and fractions of 0.45 mL were collected. Molecular weight standards used were lactate dehydrogenase (136 000), bovine serum albumin (67 000), ovalbumin (43 000), and myoglobin (17 200). The average molecular weights ± standard deviations obtained from three independently standardized columns were the following: AMP1, 92 300 ± 5500; AMP2, 86 500 ± 3800; AMP3, 83 300 ● 3000; AMP4, 61 300 ± 2900; ENP1, 67 600 ± 200.

The AMP2 isozyme purified by HA chromatography gave an intense protein band which corresponded to the enzyme-activity stain on nondenaturing polyacrylamide gel electrophoresis (Figure 3a). The $NaDodSO_4$–polyacrylamide gel electrophoresis pattern of the AMP2 preparation (Figure 3b) showed a major protein band with a molecular weight of 88 000, a value which corresponded closely to the native molecular weight of the isozyme as obtained from Sephadex G-150. Two other protein bands of less intensity and having molecular weights of 67 000 and 52 000 were also present in the AMP2 preparation. The same protein profile on $NaDodSO_4$ gels was obtained from two independent AMP2 purifications, one from maize line W64A and the other from maize line T21. We conclude that the major band on $NaDodSO_4$–polyacrylamide gel electrophoresis is AMP2 protein based upon correspondence of protein and AMP2 activity on nondenaturing gels and upon correlation of OD_{280} and AMP2 activity from HA chromatography (Figure 2b). Thus, AMP2 is a monomer of ~88 000 daltons.

The subunit compositions of two other peptidases, AMP3 and ENP1, which were also purified by HA chromatography could not be determined definitively by $NaDodSO_4$–polyacrylamide gel electrophoresis. Since these preparations were not as pure as AMP2, it was not possible to identify which of the several bands on $NaDodSO_4$–polyacrylamide gel electrophoresis corresponded to peptidase protein.

Comparative Substrate Properties of Aminopeptidases. Aminopeptidase isozymes purified at least through the gel filtration step were used to determine the specificities of the isozymes for amino acid–NA substrates. Each isozyme produced by nonallelic genes had its own distinct profile (Table II). AMP1 has highest activities toward the basic amino acids, arginine and lysine, while AMP2 was characterized by highest activities with the small amino acids, glycine and alanine. The patterns for isozymes 3F and 3S, which were products of allelic genes, were almost identical and were also very similar to the AMP1 profile. AMP4 was distinguished from the other aminopeptidases by its preference for naphthylamide bonds formed by the aromatic amino acids phenylalanine, tyrosine, and tryptophan. AMP isozymes were not active with derivatives of the acidic amino acids or their amides.

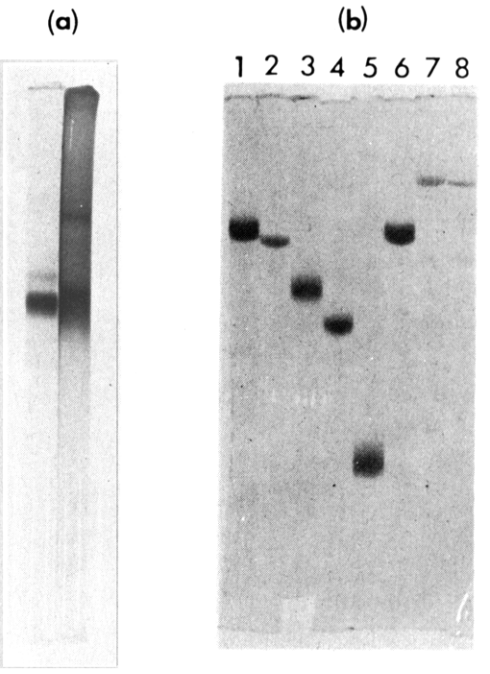

FIGURE 3: Analyses of AMP2 after HA chromatography. (a) 50-μg samples applied to nondenaturing polyacrylamide gels were stained with amido black for protein (left) or with Ala-NA (right) for enzyme activity. Enzyme activity was very intense as seen on the gel stained for Ala-NA hydrolyzing activity, and there was some trailing evident. The faint, slow-migrating activity band is probably an artifact. (b) $NaDodSO_4$–polyacrylamide gel electrophoresis for molecular weight markers and AMP2. Samples: (1 and 6) bovine serum albumin, 67 000; (2) catalase, 60 000; (3) ovalbumin, 43 000; (4) lactate dehydrogenase, 35 000; (5) lysozyme, 14 400; (7 and 8) 11 μg of AMP2, 88 000 ± 4000 for three determinations. Phosphorylase *b*, 94 000, was also used as a marker in other runs.

Table II: Relative Activities[a] of Aminopeptidase Isozymes toward the Given Amino Acid–β-Naphthylamide Substrate

AMP1-F		AMP2-S		AMP3-S		AMP3-F		AMP4-S	
Lys	151	Gly	131	Lys	109	Lys	108	Phe	244
Arg	100	Ala	100	Arg	100	Arg	100	Tyr	189
Leu	59	Met	46	Met	85	Met	98	Leu	100
Met	43	Phe	24	Leu	59	Leu	62	Trp	92
Orn	34	Arg	21	Phe	43	Phe	42	Met	83
Trp	18	Ser	18	Tyr	26	Tyr	20	Gly	8.3
Phe	16	Trp	18	Trp	16	Trp	15	Ala	6.0
Ala	12	Tyr	16	Ala	12	Ala	13	Pro	4.7
Tyr	9.3	Val	12	Orn	5.2	Orn	5.2	Val	2.2
Ser	2.4	Leu	11	Ser	2.0	Gly	1.9	Hyp	1.9
Val	1.3	Lys	10	Gly	1.5	Ser	1.5	Ile	1.6
Gly	1.1	Orn	10	Val	1.3	Val	1.4	Ser	1.4
His	<1.0	Ile	9.3	Ile	<1.0	Ile	1.1	Arg	1.2
Thr	<1.0	His	3.5	His	<1.0	His	1.1	Lys	<1.0
Hyp	<1.0	Thr	2.1	Thr	<1.0	Thr	<1.0	Orn	<1.0
Asn	<1.0	Pro	2.0	Pro	<1.0	Pro	<1.0	His	<1.0
Ile	0.0	Asn	1.9	Asn	<1.0	Asn	<1.0	Thr	<1.0
Pro	0.0	Hyp	<1.0	Hyp	<1.0	Hyp	<1.0	Asn	0.0
Asp	0.0	Asp	0.0	Asp	0.0	Asp	0.0	Asp	0.0
Glu	0.0	Glu	0.0	Glu	0.0	Glu	0.0	Glu	0.0

[a] The commonly used substrate (Arg-NA, Ala-NA, or Leu-NA) is given the value 100 for each isozyme. Values are the means of at least two independent determinations. Absolute activities were expressed as micromoles of substrate hydrolyzed per hour per milliliter. All assays were performed with 0.34 mM substrate in 0.1 M potassium phosphate, pH 7.5.

Apparent K_m values determined for the aminopeptidases with the three standard substrates and for AMP4 with Phe-NA

Table III: Apparent K_m Values with Amino Acid-NA Substrates[a]

enzyme	$K_m \times 10^5$			
	Ala-NA	Arg-NA	Leu-NA	Phe-NA
AMP1-F	127 ± 68	29 ± 10	22 ± 10	–
AMP2-S	26 ± 6.4	20 ± 7.7	9.0 ± 3.8	–
AMP3-S	122 ± 72	14 ± 2.5	16 ± 9.2	–
AMP4-S	20 ± 4.5	–	8.6 ± 3.4	32 ± 12

[a] Assays were performed in 0.1 M potassium phosphate, pH 7.5. Each value is the mean of three determinations ± one standard deviation. (–) Not determined.

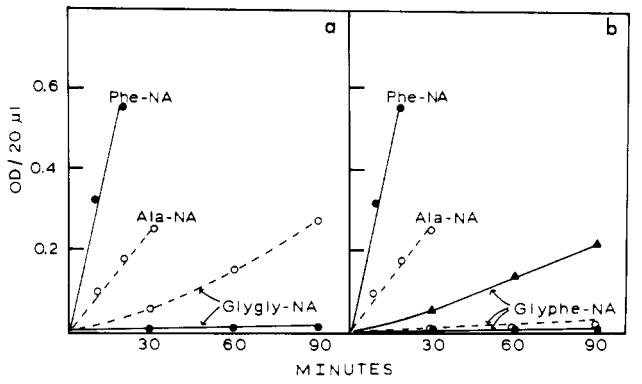

FIGURE 4: Hydrolysis of GlyGly-NA (a) and GlyPhe-NA (b) by AMP2 and AMP4. Isozyme 4S with the indicated substrate (●); isozyme 2S with the indicated substrate (○); a combination of 2S + 4S, 20 µL each, with GlyPhe-NA substrate (▲).

are shown in Table III. Enzyme purified at least through the gel filtration step was used. AMP4 had a higher relative activity with Phe-NA than with Leu-NA, although the K_m for Phe-NA was higher than that for Leu-NA. Similarly AMP2 had a higher activity for Ala-NA but the K_m was lower with Leu-NA. Pronounced substrate inhibition was observed for AMP1 at >0.34 mM Arg-NA concentration.

GlyGly-NA was hydrolyzed by AMP2 with the liberation of free naphthylamine (Figure 4a). Two mechanisms for the hydrolysis were possible:

$$\text{GlyGly-NA} \rightarrow \text{Gly} + \text{Gly-NA}$$
$$\text{Gly-NA} \rightarrow \text{Gly} + \text{NA} \quad (1)$$
$$\text{GlyGly-NA} \rightarrow \text{GlyGly} + \text{NA} \quad (2)$$

The nonlinearity of the reaction rate indicated that the reaction occurred as in mechanism 1. Further support that the enzyme cleaved sequentially from the amino-terminal end is shown in Figure 4b. Individually, isozymes 2S and 4S hydrolyzed GlyPhe-NA very slowly, if at all. Together, however, they acted synergistically to markedly increase the rate of hydrolysis. Our interpretation is that the substrate was hydrolyzed predominantly in the following manner:

$$\text{GlyPhe-NA} \xrightarrow{2S} \text{Gly} + \text{Phe-NA}$$
$$\text{Phe-NA} \xrightarrow{4S} \text{Phe} + \text{NA}$$

The results were consistent both with an aminopeptidase mechanism of action for AMP2 and with the comparative substrate specificities for isozymes 2S and 4S, as shown in Table II.

Aliquots of AMP2 purified through HA chromatography were run on polyacrylamide disc gels and stained for Ala-NA activity, as the control, and for hydrolytic activity on dipeptide substrates by using an amino acid oxidase coupled reaction (Lewis & Harris, 1967). The following substrates were tested and all were hydrolyzed by AMP2: AlaLeu, LeuAla, GlyLeu, GlyPhe, and GlyGlyLeu. Purified isozyme 3F was tested with

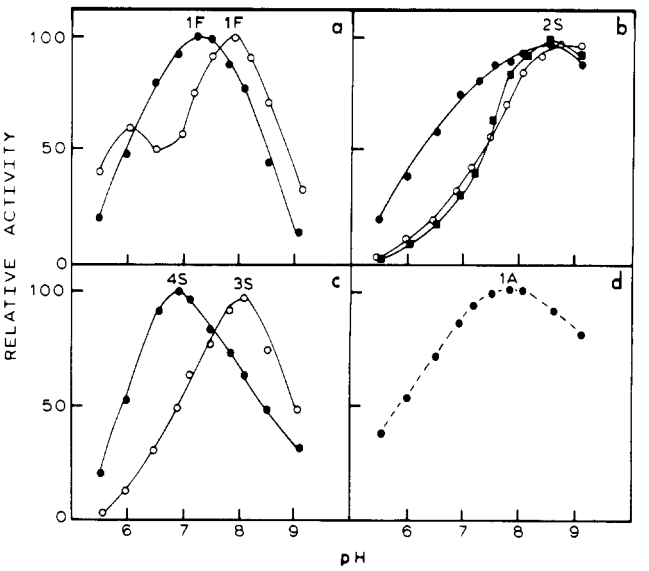

FIGURE 5: Dependence of peptidase activities on pH. Assays were performed in 0.15 M potassium phosphate at the given pH. (a–c) Aminopeptidase isozymes as indicated were assayed with Leu-NA (●), Arg-NA (○), or Ala-NA (■). (d) Endopeptidase activity with BANA.

these peptides also and staining could be detected only with LeuAla as the substrate.

Peptidase activities as a function of pH are shown in Figure 5. AMP1 had optima near pH 7.3 and 7.9 for Leu-NA and Arg-NA, respectively. At pH 6, AMP1 also had a second peak which occurred only with Arg-NA substrate. AMP2 had a broad optimum near pH 8.5 for Ala-NA, Arg-NA, and Leu-NA. AMP3 had an optimum at pH 8.1 with Arg-NA but did not possess a second peak at pH 6 as did AMP1. AMP4 had an optimum at pH 6.9 with Leu-NA. In comparison to the aminopeptidases, maize endopeptidase is also optimally active in the slightly alkaline range. ENP1 had a broad peak from pH 7.5 to pH 8.0.

Comparative Effects of Sulfhydryl Reagents, Metals, and Chelators. The effect of some reagents on aminopeptidase and endopeptidase activities is shown in Table IV. The sulfhydryl-complexing reagent *N*-ethylmaleimide gave partial inhibition of some isozymes, and *p*-mercuribenzoate (*p*MB) at 0.1 mM inhibited >50% of each enzyme activity. Reducing reagents did not enhance isozyme activities; in fact, dithiothreitol at 1 mM partially inhibited AMP3. Cyanide and citrate were not inhibitory. ENP was completely inhibited by TLCK (tosyllysine chloromethyl ketone) at 0.01 mM but only partially inhibited by TPCK (tosylphenylalanine chloromethyl ketone) at a 10-fold higher concentration. Phenylmethanesulfonyl fluoride (PMSF) was not inhibitory to AMP or ENP.

EDTA and 1,10-phenanthroline strongly inhibited AMP1 and AMP3 (Table V). With a 45° C incubation, AMP2 was also inhibited by 1 mM phenanthroline but not by EDTA (Figure 6). AMP4 was considerably more heat labile than AMP2 and was protected to some degree by the inclusion of EDTA or phenanthroline.

Of the metal ions tested, Zn^{2+} at low concentrations strongly inhibited the AMP isozymes but not ENP (Table V). Cu^{2+} and Hg^{2+} were also strongly inhibitory to aminopeptidases (data not shown). Ca^{2+}, and to some extent Mg^{2+}, at 5–10 mM concentration stimulated ENP. Under certain assay conditions 10 mM Ca^{2+} enhanced ENP activity by four- to fivefold. This enhancement was obtained when BAPNA substrate was used rather than BANA and when a lower Tris buffer concentration, 30 mM as opposed to 100 mM, was used

Table IV: Comparative Effects of Some Reagents on Peptidase Activities[a]

addition	concn (mM)	% rel act. AMP1-F	AMP2-S	AMP3-S	AMP4-S	ENP1-A
control		100	100	100	100	100
iodoacetamide	1.0	86	99	88	102	105
N-ethylmaleimide	1.0	61	92	69	89	48
*p*MB	0.1	16	48	48	28	20
mercaptoethanol	1.0	102	93	96	105	94
dithiothreitol	1.0	88	105	66	104	94
KCN	1.0	110	93	109	97	101
citrate	10.0	120	90	123	96	116
TLCK	0.1	91	96	85	97	0
TLCK	0.01	98	97	87	104	0
TPCK	0.1	110	89	64	104	63
PMSF	0.1	93	98	87	106	100

[a] Reagents were adjusted to pH 7.5 in 0.1 M Tris-HCl. Enzyme was incubated with twice the final concentration of reagent for 1 h prior to the addition of substrate. L*p*NA was used as the substrate for all aminopeptidases and BAPNA for endopeptidase since some of the compounds tested interfere with the diazotization reaction used in the assay of naphthylamine. Assays were performed in triplicate, and results are the average of at least two independent determinations.

Table V: Comparative Effects of Chelators and Metal Ions on Peptidase Activities[a]

addition	concn (mM)	% rel act. AMP1-F	AMP2-S	AMP3-F	AMP4-S	ENP1-A
control		100	100	100	100	100
EDTA	0.5	2	106	17	107	103
1,10-phenanthroline	0.5	11	74	4	108	83
$MgCl_2$	5.0	110	92	91	91	121
$CaCl_2$	5.0	110	88	79	86	130
$MnSO_4$	0.05	99	88	89	108	105
$ZnSO_4$	0.05	39	14	22	13	98

[a] The substrates commonly used for each enzyme were employed in these assays, i.e., Arg-NA for 1F and 3F, Ala-NA for 2S, Leu-NA for 4S, and BANA for 1A. Enzyme was incubated with twice the final concentration of reagent for 1 h prior to the addition of substrate. Assays were performed at pH 7.5 with 87.5 mM Tris-HCl.

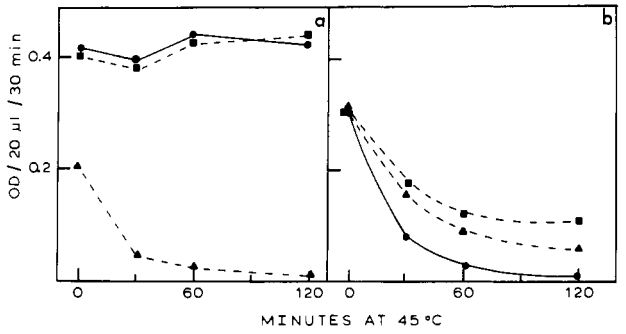

FIGURE 6: Effects of EDTA and 1,10-phenanthroline on AMP2S (a) and AMP4s (b). Control (●); 1 mM EDTA (■); 1 mM 1,10-phenanthroline (▲). Assays were performed with Ala-NA substrate for AMP2 and Leu-NA for AMP4 in 100 mM Tris-HCl, pH 7.5. Enzyme was incubated with reagent at twice its final concentration for the indicated time in a 45 °C water bath. Tubes were immersed in H_2O for 2 min and then assayed at room temperature upon addition of substrate. Each time point was assayed in triplicate and results are the average of two experiments.

in the assay. A similar effect of Ca^{2+} and Mg^{2+} on AMP1 activity was observed although it was not pronounced in the results of Table V because Arg-NA was used as the substrate in those determinations. Up to two- to fourfold stimulation by 10 mM Ca^{2+} was found if Leu-NA or L*p*NA were used as substrate in a low-concentration Tris buffer. Ca^{2+} may induce a conformational change or in some way help to position the noncharged side chain of Leu-NA or L*p*NA in the substrate binding site.

Discussion

Use of three maize lines with different genotypes for the peptidases enabled us to preparatively separate the isozymes with DEAE-Sephadex. For enzyme characterization studies, isozymes purified at least through the Sephadex G-150 gel filtration step were used. At this point, the peptidases were separated from one another except for AMP3S and ENP1A. We achieved separation of AMP3S and ENP1A by HA chromatography and also further purified AMP2S by this method. Attempts to further purify AMP4 by HA chromatography or isoelectric focusing resulted in high activity losses. Additional purification of AMP1 was not attempted. All purified peptidases, including AMP4, could be stored in 50% ethylene glycol at −20 °C for more than 1 year with 95–100% maintenance of activities. Starch gel electrophoresis of these preparations showed that no changes in the electrophoretic mobilities had occurred and that each isozyme, with the exception of AMP3, appeared as a single band of enzymatic activity. Isozymes 3F and 3S each consist of a main band and up to three additional bands which migrate more anodally than the main band and which are often diffuse. This pattern appears for AMP3 in both crude extracts and in purified preparations (Vodkin & Scandalios, 1979). Because the maize peptidases showed similarities in developmental expression (Vodkin & Scandalios, 1979) and because the aminopeptidases had overlapping substrate specificities, we concluded that it would be more informative to examine the peptidases as a group rather than to attempt purification and extensive characterization of only one of the peptidases. Although all of the isozyme activities are highly purified and have been effectively separated from one another, they are not homogeneous; therefore, the kinetic and inhibitor data which are presented for comparative purposes should be viewed in this light.

Comparisons among the nonallelic AMP isozymes showed that each was unique in its properties although they shared

many overall similarities. All were inhibited by *p*MB and by the divalent cations Hg^{2+}, Cu^{2+}, and Zn^{2+}. AMP4, however, was noticeably distinguished from the others in several respects. It had essentially no activity with Arg-NA and Lys-NA substrates, unlike AMP1, AMP2, and AMP3. The 1% relative activity of AMP4 with Arg-NA and Lys-NA was possibly due to trace contamination by AMP3. In comparison with the other aminopeptidases, AMP4 had the lowest molecular weight (61 000), as determined by gel filtration, and the lowest optimum pH (6.9) as determined with Leu-NA as the substrate. AMP1, AMP2, and AMP3 were inhibited by 1 mM or lower concentrations of EDTA or 1,10-phenanthroline, but AMP4 was not inactivated by these chelators. AMP4 had the highest activity upon the initial separation of the isozymes on DEAE-Sephadex, but AMP4 was unstable to HA chromatography, to removal of ampholytes after isoelectric focusing, and to both starch gel and polyacrylamide gel electrophoreses. It is possible that these instabilities were caused by trace contamination with metal ions which were found to strongly inhibit AMP4. Buffers containing EDTA may protect against AMP4 activity losses during purification.

AMP4 was very similar to barley grain and pea seed aminopeptidases which have been partially purified and characterized (Kolehmainen & Mikola, 1971; Elleman, 1974). Both the barley and pea enzymes have neutral pH optima and prefer substrates with hydrophobic residues, especially Phe-NA, Tyr-NA, and Met-NA. They have no activity with Arg-NA and Lys-NA. The barley enzyme is 65 000 daltons and is slightly activated by EDTA. The pea enzyme (designated AP1) is 58 000 daltons and is not inhibited by EDTA or 1,10-phenanthroline. Both enzymes are sensitive to *p*MB but not to diisopropyl fluorophosphate. It is also interesting to note that both barley and pea have multiple forms of the aminopeptidases as does corn. In pea seeds a second enzyme, AP2, with higher activities toward Ala-NA, Arg-NA, and Lys-NA was also partially purified and characterized. AP2 resembles the corn enzymes AMP1, AMP2, and AMP3 in that it is inhibited by *p*MB and is also sensitive to 1,10-phenanthroline. Whether the lack of inhibition of AMP4 by chelators reflects a lack of metal ion involvement in the enzyme mechanism can only be determined by further studies. However, insensitivity to 1,10-phenanthroline is a distinguishing characteristic of the pea and corn enzymes which have highest activities with hydrophobic aminoacyl residues.

The present report on the properties of maize peptidases is the first to have utilized highly purified enzyme preparations. In initial studies (Ott & Scandalios, 1976) with crude enzyme preparations or electrophoretically separated isozymes, we reported that AMP3 had higher relative activity with Leu-NA than with Arg-NA and that AMP4 had appreciable activity with Arg-NA. In fact, AMP3 activity is higher with Arg-NA than with Leu-NA, and AMP4 has no activity with Arg-NA as shown in Table II. The discrepancies arose from a lack of total separation between AMP3 and AMP4 in the electrophoretically separated isozyme preparations used in the earlier studies. The close migration of AMP3 and AMP4 on gels and the diffuse nature of AMP3 led to considerable contamination between the two isozymes and thus to a blurring of the distinction between them. Using highly purified and cleanly separated preparations, we have found that AMP4 is noticeably distinct from AMP3 in a number of properties.

AMP2, the isozyme which is expressed only in the immature maize endosperm, was most active with the NA derivatives of the smallest amino acids, i.e., Gly-NA and Ala-NA. AMP2 is a monomeric protein of ~88 000 daltons. It had true aminopeptidase activity, i.e., it cleaved GlyGly-NA sequentially from the amino-terminal end, and it was active in hydrolyzing dipeptide substrates. The more rapid hydrolysis of GlyPhe-NA by a combination of AMP2 and AMP4 than by either alone is a complementary action of the isozymes which is likely to occur in vivo with natural peptide substrates.

The neutral to slightly alkaline pH optima of maize aminopeptidases and the apparent K_m values were similar to those reported for other plant naphthylamidases (Tulley & Beevers, 1978; Salmia & Mikola, 1976; Kolehmainen & Mikola, 1971; Elleman, 1974).

The differential effects of TLCK and TPCK on ENP activity strongly indicate that the charged TLCK is an active site directed inhibitor of ENP as it is of trypsin (Shaw et al., 1965). The enhancement of AMP1 and ENP activities by high concentrations of Ca^{2+} and Mg^{2+} was affected by the Tris concentration and was more pronounced with *p*-nitroaniline derivatives. High concentrations of $(NH_4)_2SO_4$ (0.25 M) also increased ENP activity. The mechanism of Ca^{2+} enhancement possibly involves a conformational shift in the enzyme. Ca^{2+} and various buffers of high ionic strength have been shown to activate and stabilize trypsin by inducing a conformational rearrangement in the enzyme (Sipos & Merkel, 1970).

While naphthylamidase activities in pea have been studied to some extent for genetic control, developmental expression, and biochemical properties (Scandalios & Espiritu, 1969; Scandalios & Campeau, 1972; Elleman, 1974), the maize enzymes have been more extensively investigated with respect to the above parameters. These peptidases appear to have a general metabolic function in cell metabolism and do not have a direct role in the hydrolysis of reserve proteins during germination (Vodkin & Scandalios, 1979). However, they likely have the function of hydrolyzing small peptides resulting from intracellular protein turnover or from peptide transport. Although a major impediment would be obtaining large amounts of homogeneous preparations of each isozyme, the maize peptidases present a potentially fruitful system for determining more detailed comparisons of substrate specificities and other physicochemical parameters of the enzymes. Such studies would aid in understanding evolutionary and structure–function relationships among peptidases.

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Immunologically Specific Complexes of Chromosomal Nonhistone Proteins with Deoxyribonucleic Acid in Chicken Erythroid Nuclei†

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ABSTRACT: Erythroid cell-specific antisera capable of detecting chromosomal nonhistone protein–DNA complexes were obtained by injecting rabbits with dehistonized chicken reticulocyte chromatin. The specific antigenic nonhistone protein–DNA complexes were relatively inaccessible to the antiserum in isolated erythrocyte chromatin. However, isolation of chromatin from cells at earlier stages of erythropoiesis or treatment of isolated erythrocyte chromatin with polyanions or phenylhydrazine provided materials with significantly increased immunogolical reactivity. The altered activity was caused by changes in conformation occurring at two levels: a specific one, determined by chromosomal nonhistone proteins, and a more general one, determined by histones. Immunological examination of fractionated products obtained from limited nuclease digestion revealed the localization of the antigenic complexes in the nuclease-resistant, large fragments of erythroid chromatin. The nuclease-resistant DNA isolated from the immunologically reactive fragments migrated in gel electrophoresis as a diffuse band of between 1000 and 2000 base pairs. No preferential accumulation of globin-specifying DNA sequences could be found in this nuclease-resistant DNA. The protein fraction containing the immunologically cell-specific complexes in chicken erythrocyte chromatin was glycosylated and moderately acidic (by amino acid analysis) with an electrophoretically determined M_r of ~90 000.

Introduction of immunological methods to the studies on chromosomal proteins opened a new chapter in chromatin research. Antibodies to histones and other chromatin components are progressively applied to the investigations on chromatin structure and function (Bustin, 1978; Silver & Elgin, 1978). It was first shown by Spelsberg et al. (1971) that chromatin can be selectively dehistonized in concentrated salt–urea buffers at pH 6 or 5. When such dehistonized chromatins were used as immunogens, cell- and tissue-specific antibodies could be elicited (Chytil & Spelsberg, 1971; Wakabayashi & Hnilica, 1973). Presently, antibodies of various specificity to whole or dehistonized chromatin as well as to nuclear nonhistone protein fractions are being used to study the cell, species, and tumor specificity of the respective antigens [reviewed in Hnilica et al. (1978), Silver & Elgin (1978), and Hnilica & Briggs (1979)].

Immunization with dehistonized chicken reticulocyte chromatin produced two types of antisera. One, observed only rarely, was specific for reticulocyte chromatin and did not react significantly with dehistonized, fractionated, or sheared erythrocyte chromatin (Hardy et al., 1978). The other could be obtained more frequently and reacted extensively with chicken reticulocyte chromatin and also to a lesser, but significant, extent with isolated erythrocyte chromatin (Krajewska et al., 1979). The protein component of the nonhistone protein–DNA complex responsible for the latter reaction specificity was isolated and partially characterized. Examination of the immunological reactivity and accessibility of such complexes revealed information about their likely intrachromosomal localization as related to some general features of chromatin organization in maturing, late stage chicken erythroid cells.

Experimental Procedures

Materials. Adult male chickens (Leghorn, Dekalb strain) were made anemic by daily injections of 1% neutralized phenylhydrazine (10 mg/kg), followed by daily bleeding until polychromatic primitive erythrocytes represented at least 95% of the total circulating red blood cells (Hardy et al., 1978). In a separate schedule, anemia (70% of polychromatic primitive erythrocytes) was obtained by extensive daily bleeding alone. Mature erythrocytes were obtained by cardiac puncture or by decapitation of the untreated animals. Blood was collected in ice-cold 0.15 M saline–15 mM sodium citrate solution containing 0.01% heparin. Erythroid nuclei were prepared either by the method of Evans & Lingrel (1969) with the addition of 0.5% Triton X-100 wash or by nitrogen cavitation (Shelton et al., 1976). Both methods gave essentially identical results. Isolated nuclei were purified by centrifugation through 1.8 M sucrose in 50 mM Tris-HCl,[1] pH 7.4, 24 mM KCl, 5

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