Iodination of *Xenopus laevis* Histone F2a1 in Chromatin†

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ABSTRACT: The reaction of calf thymus and *Xenopus laevis* histones with radioactive iodine has been studied under various conditions that affect chromatin structure. All histones from both species contain at least one tyrosine residue and, in a denaturing solvent, all of the histones react with iodine. Histone F2a1 has been studied in detail. Calf thymus F2a1 is known to contain four tyrosyls and all four react with iodine. In high voltage paper electrophoresis, the tyrosine-containing peptides from calf co-migrate with those from *Xenopus* F2a1, suggesting that the amino acid sequence is strongly conserved between these two species. Therefore, the published calf thymus F2a1 sequence has been used to order the *Xenopus* F2a1 peptides within the molecules. When gently isolated native chromatin is iodinated in a low ionic strength medium, 60% of the radioactivity in F2a1 is in tyrosyl 88, 30% in tyrosyl 51, and tyrosyl 72 and 98 have almost no radioactivity. Reagents which remove the protein from the DNA (2 M NaCl) or partially disrupt protein tertiary structure (5 M urea) increase the reactivity of each of the four tyrosyls five- to tenfold, suggesting that all four are protected about equally by the overall folding of the chromatin. Isolated F2a1 iodinated in the presence of 10 M urea shows uniform labeling in each of the four peptides, suggesting that tyrosyl 72 and 98 are afforded some protection solely by protein–protein interactions. The stepwise removal of histones in increasing NaCl concentrations differentially increases the availability of each F2a1 tyrosyl. The preferential exposure of tyrosyl 88 coincides with the removal of the majority of F1 histones at 0.5 M NaCl while the gradual and stepwise increase in reactivity of tyrosyl 51, 72, and 98 correlates with the gradual removal of histones other than F1. Radioactive iodination of chromatin has been shown to be a sensitive probe for detecting changes in the association state (or conformation) of histone F2a1.

Histones interact with DNA and with each other to produce ordered subunit structure in chromatin (Olins and Olins, 1974; Hewish and Burgoyne, 1973; Weintraub and Van Lente, 1974; Sahasrabuddhe and Van Holde, 1974; Noll, 1974; D'Anna and Isenberg, 1974; Kornberg and Thomas, 1974). A variety of reagents have been utilized in attempts to elucidate the details of these interactions.

Selective labeling agents such as DTNB[1] (Hyde and Walker, 1974) and acetic anhydride (Simpson, 1971; Malchy and Kaplan, 1974) have been used to detect buried and exposed amino acid residues in histones. Histone–histone association has been examined by use of several cross-linking agents (Olins and Wright, 1973; Martinson and McCarthy, 1975; Van Lente et al., 1975; Kornberg and Thomas, 1974; Chalkley and Hunter, 1975), and Weintraub and Van Lente (1974) have used trypsin digestion in attempts to analyze the topography of the chromatin subunit.

The reaction of radioactive iodine with protein tyrosyl residues is another reaction which can be used to study protein interactions as shown by previous studies on insulin (Hamlin and Arquilla, 1974) and on ribosomes (Litman and Cantor, 1974). In this paper we have used the iodination reaction to probe chromatin structure with special emphasis on the reaction of histone F2a1.

Methods

I. Chromatin and Histone Preparation. Xenopus laevis chromatin and histones were prepared from an established cell line originally derived from *Xenopus* kidney (Brown and Weber, 1968) and grown in large roller bottles at room temperature in 100 ml of modified Waymouth (Gibco) containing 10% fetal calf serum. Chromatin was prepared from cultured cells by modification of the method of Hancock (1974). Cells were scraped off in growth medium and centrifuged into a pellet at room temperature. Cells were then suspended in 0.2 mM phosphate buffer at pH 7.4, 20% sucrose, and centrifuged again. All subsequent operations were performed at 4 °C. The cells were homogenized gently, then, while Vortexing, the solution was made to 0.25% NP-40 (Nonidet P-40, Shell Chemicals), 0.1 M EDTA by diluting with a twofold concentrated solution of NP-40 and EDTA. The lysed cells were then layered on top of 0.2 mM phosphate buffer (pH 8.5), 20% sucrose and centrifuged in a swing-out rotor at 5000 rpm, 10 min. The pellet, which contained swollen nuclei devoid of a limiting membrane, was washed several times (in phosphate–sucrose, pH 8.5) until the chromatin was clean of other cellular material as observed by phase-contrast microscopy. Finally, the pellet was taken-up in 5 mM Tris, 1 mM EDTA and homogenized with 10 strokes in a Dounce homogenizer. The A_{320}/A_{260} ratio of the final preparation was between 0.1 and 0.2.

Histones were isolated from purified chromatin by extracting with 0.4 N H_2SO_4 on ice for 30 min. After centrifugation at 10 000 rpm for 10 min, the supernatant was combined with four volumes of absolute ethanol and precip-

† From the Department of Embryology, Carnegie Institution of Washington, Baltimore, Maryland 21210. *Received November 11, 1975.* This work was in partial fulfillment of the requirements for the Ph.D. degree for Sandra Lyn Biroc from the Johns Hopkins University and was partially supported by NIH training Grant no. HD-139.

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[1] Abbreviations: EDTA, ethylenediaminetetraacetate; F1, I; F2a1, IV; F2a2, IIb1; F2b, IIb2; F2c, V; F3, III; Temed = *N,N,N′,N′*-tetramethylethylenediamine; TosPheCH$_2$Cl, L-1-tosylamido-2-phenyl ethyl chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); CNBr, cyanogen bromide; sp ac, specific activity.

itated at −20 °C for 16 h. The white histone precipitate was vacuum dried and dissolved in either distilled water, 10 M urea, or 0.1 N acetic acid.

Calf thymus chromatin was isolated as described by Bonner et al. (1968).

II. Acrylamide Gel Electrophoresis. A. Acid–Urea Gels. Acid–urea disc acrylamide gels were prepared according to the method of Panyim and Chalkley (1969a) except the urea concentration was varied depending upon the peptides and proteins to be separated. Acrylamide concentration was 15%, bisacrylamide 0.1%, acetic acid 5.4%, Temed 0.5%, and ammonium persulfate 0.125%. The tube gels of dimensions 0.6 × 25 cm were loaded with 10–30 μg of protein and electrophoresed at 200 V (constant voltage) for 22 h. Acrylamide gels containing 2.5 M urea gave the best separation of calf thymus histones, while gels containing 1 M urea gave the best separation for *Xenopus laevis* histones. Cyanogen bromide fragments of histone F2a1 were run on 0.6 × 12 cm gels containing 6.25 M urea at 4 mA/gel (constant current) for 4 h without pre-electrophoresis. Under these conditions the marker dye, 0.01% basic fuchsin, electrophoresed just to the end of the tube and histone F2a1 migrated to about the middle. The gels were stained in 0.1% Amido Schwartz, 20% ethanol, 7% acetic acid for 16 h and then in 10% acetic acid, 5% ethanol until destained. The de-stained gels were scanned on a Joyce-Loebl microdensitometer.

For determination of radioactivity in ^{125}I-labeled preparations, the gel was fractionated into 1.0-mm slices on a Gilson gel slicer using 30% acetic acid, 0.03% bovine serum albumin in distilled water as the flushing solvent. Fractions were counted in a γ counter (Packard) at an efficiency of 65%.

B. Sodium dodecyl sulfate slab gels were prepared as described by Laemmli (1970) except that the bisacrylamide was lowered to 0.2%. Slab gels were stained in 12.5% Cl_3CCOOH containing 0.01% Coomassie G-250 (Diezel et al., 1972) and then destained in 5% ethanol, 10% acetic acid. The gels were photographed while still wet, then dried down onto a piece of filter paper by water aspiration suction and steam heat by a modification of the method of Fairbanks et al. (1965), and then autoradiographed with x-ray film.

III. Cyanogen Bromide Cleavage of Uniformly Iodinated Calf Thymus F2a1. Purified calf thymus F2a1 (a gift from Dr. D. M. Fambrough) was dissolved in 10 M urea at a concentration of 10 mg/ml and trace labeled with ^{125}I. The protein to iodine ratio was 50:1 to decrease the amount of di-iodinated reaction products and to avoid histidine labeling. Under these conditions, greater than 90% of the radioactivity was present as mono-iodotyrosine. Unreacted ^{125}I was separated from ^{125}I-labeled F2a1 on a Bio-Gel P-10 desalting column.

The iodinated calf thymus F2a1 was dissolved in 70% formic acid in a total volume of 0.4 ml, 20–30 mg of solid cyanogen bromide was added (about 50-fold molar excess over the protein), and the mixture was allowed to react at room temperature for 16–24 h. The cleavage was more complete if the protein was first treated for 40 h with 40% 2-mercaptoethanol to reduce any oxidized methionine caused by the treatment of the protein with Chloramine-T. The entire reaction mixture was diluted to 4 ml with distilled water and lyophilized to dryness. The residue was taken up in 100 μl of 10 M urea and applied to an acid–urea acrylamide gel containing 6.25 M urea and 15% acrylamide.

IV. Tryptic Peptide Maps. Purified histone F2a1 was extracted from acrylamide gel slices by incubating each minced slice in 0.3 ml of 30% acetic acid, 0.03% bovine serum albumin at 4 °C overnight. The liquid was removed, taking care to avoid pipetting up any gel particles, and lyophilized to dryness. The residue was dissolved in 100 μl of 0.1 M ammonium bicarbonate, pH 8.5–9.0, 1 M urea, and 5 μl (10 μg) of TosPheCH_2Cl-trypsin (Worthington) was added. The digestion was at 37 °C for 24 h, over which time 5 μl of TosPheCH_2Cl-trypsin was added twice more. The entire sample was spotted on an origin line 10 cm from the end of Whatman 3 MM paper (20 × 68 cm) using 10-μl aliquots and drying with a warm hair dryer. The paper was moistened with pH 3.5 buffer (5% acetic acid, 0.5% pyridine), blotted dry, and electrophoresed at 3000 V for 2 h. The paper was dried and autoradiographed with x-ray film. For two-dimensional electrophoresis, strips (5 × 68 cm) were electrophoresed in the first dimension, dried, and autoradiographed to determine the position of the radioactive peptides. Each was then sewn onto a large sheet of 3 MM paper and electrophoresed in the second dimension at pH 6.5 (10% pyridine, 0.3% acetic acid) or pH 1.9 (2.5% formic acid, 8.7% acetic acid).

To determine the amount of iodotyrosine and diiodotyrosine in the protein, a portion of each histone sample was dissolved in 100 μl of distilled water, digested with 10 μg of Pronase (Sigma) for 5 h at 37 °C, and electrophoresed at pH 1.9 for 45 min at 4000 V. Iodotyrosine and diiodotyrosine are well separated from each other and migrate slower than tyrosine. The retarded migration is probably due to altered pK_a and increased molecular weight. Less than 1% iodohistidine was ever found.

V. Iodination of Chromatin. Chromatin was reacted with radioactive iodine by the method of Hunter and Greenwood (1962). Additions to the reaction were made in the following order: (1) 100 μl of chromatin (1 mg/ml of protein, ca. 10 nmol of histone) in 5 mM Tris (pH 8.0), 1 mM EDTA; (2) 100 μl of water, 10 M urea and 4 M NaCl, or 10 M urea and 23 mg NaCl; (3) 5 μl of 0.12 mM KI (0.6 nmol); (4) 5 μl of 1 mM L-tyrosine (5 nmol); (5) 1 μl of 200 μCi/ml of ^{125}I in 0.1 N NaOH (New England Nuclear) (6.4 pmol); (6) 10 μl of 7 mM Chloramine-T (70 nmol). The reaction was allowed to proceed 15 min at room temperature then was stopped by the addition of 10 μl of 21 mM sodium metabisulfite (210 nmol). The DNA and nonhistone proteins were precipitated by the addition of 25 μl of 4 N H_2SO_4. The acid-treated reaction mixture was allowed to sit on ice for 30 min and then centrifuged at 10 000 rpm for 15 min.

The entire supernatant (250 μl) was applied to a Bio-Gel P-10 column (1.5-ml bed volume) to separate iodinated histone from unreacted iodine and iodotyrosine.

The column was equilibrated in 30% acetic acid and, to avoid loss when desalting small amounts or protein, 1 mg of bovine serum albumin was passed through the column first. Recovery of radioactivity from the Bio-Gel P-10 column was quantitative. Histones eluted at the void volume and were further analyzed by electrophoresis in either sodium dodecyl sulfate or acid–urea acrylamide gels. Free iodine and iodotyrosine eluted at the included volume. A portion of the included volume was rechromatographed on Bio-Gel P-2 (25-ml bed volume equilibrated with 50 mM ammonium acetate, pH 5). On Bio-Gel P-2 free iodine eluted at the included volume but iodotyrosine eluted at 150% of the included volume. Recovery of iodotyrosine was 80%.

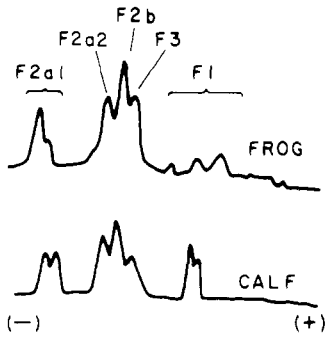

FIGURE 1: Densitometer tracing of acid–urea acrylamide gels comparing *Xenopus* and calf thymus histones. Gels (0.6 × 25 cm) were prepared containing either 1.0 or 2.5 M urea and pre-run for 16 h at 0.5 mA/tube. About 50 μg each of *Xenopus* or calf thymus histone in 5 M urea, 0.01% basic fuchsin was applied to the gel, electrophoresed for 22 h at room temperature, and stained with Amido Schwartz. Electrophoresis was from right (+) to left (−).

VI. Calculation of Reactivity Index. The ability of each histone tyrosyl to react with iodine was expressed as a reactivity index as defined by eq 1:

$$\text{reactivity index of tyrosyl X} = \frac{\text{sp ac of X}}{\text{sp ac of free tyrosine}} \quad (1)$$

In other words, the reactivity of each peptide tyrosyl was normalized to the reactivity of free tyrosine in the same reaction. The specific activity of free tyrosine was calculated from the input amount of L-tyrosine and the radioactivity recovered from the Bio-Gel P-2 column (corrected for 80% recovery).

The specific activity of F2a1 was calculated from the input amount of histone in the chromatin and the total counts recovered from the Bio-Gel P-10 column. Histone was estimated by assuming a 1:1 weight ratio of histone to DNA, and DNA was measured by absorbance of chromatin at 260 nm. Acid extraction of the histones was quantitative in the four solvents, and no radioactivity was lost on the desalting Bio-Gel P-10 column. Radioactivity associated with each individual histone was determined either from the densitometer trace of an autoradiograph of the dried slab, gel, or by running the histones in an acid–urea disc gel and summing the radioactivity in each histone when the gel was sliced and counted. The specific activity of F2a1 was calculated assuming F2a1 to be 20% by weight of the total histone (Panyim and Chalkley, 1969b; Panyim et al., 1971) and by summing the radioactivity in the F2a1 protein peak.

The specific activity of each tyrosine-containing peptide was calculated from the radioactivity associated with each tryptic spot and from the calculated specific activity of purified F2a1. A reactivity index of less than 1.0 indicated the peptide tyrosyl was less reactive to iodine than the freely diffusible amino acid, and a ratio greater than 1.0 indicated the peptide tyrosyl had a greater reactivity than free tyrosine.

VII. Rate of Tyrosine Iodination. The rate of tyrosine iodination was monitored by the appearance of iodotyrosine absorption at 310 nm (Herriott, 1947). The reaction mixture contained 500 nmol of L-tyrosine, 120 nmol of KI, and 140 nmol of Chloramine-T in a total volume of 1.0 ml. Increased absorbance at 310 nm was measured at 10-s intervals in a Zeiss spectrophotometer for reaction in the following solvents: (1) 10 mM Tris pH 8.0; (2) 10 mM Tris, 10 M urea; (3) 10 mM Tris, 5 M urea; (4) 10 mM Tris, 5 M urea, 2 M NaCl; and (5) 10 mM Tris, 2 M NaCl. The pH of each reaction mixture was measured prior to addition of Chloramine-T which initiated the reaction. The rate was computed by the time it took to reach half maximal value; maximal value was achieved in 10 min after which time there was no further change in absorbance.

Results

Electrophoretic Comparisons of Calf Thymus and Xenopus Histones. The histones from *Xenopus* have not been studied extensively and, in particular, they have not been sequenced. Therefore, we felt it essential at the outset to compare them in some detail with their better known counterparts from calf thymus. Figure 1 compares acid-extracted histones from *Xenopus* and from calf thymus after electrophoresis in acid–urea acrylamide gels. *Xenopus* histones were best resolved in 1 M urea due to the large effect urea has on the mobility of amphibian F2a2 (Panyim et al., 1971). The mobility of F2a1, F2b, and F3 could not be distinguished between the two species at any urea concentration between 0 and 5 M. In this electrophoretic system, *Xenopus* has at least three species of F1 while calf shows the usual two bands.

The *Xenopus* histones were further identified by employing the selective extraction procedure of Johns (1964). This allowed positive identification of the bands shown in Figure 1.

Xenopus and calf histones were also compared by electrophoresis in sodium dodecyl sulfate gels (Laemmli, 1970; Figure 2). In this system also the F2a1, F2b, and F3 histones from both species have the same mobilities. *Xenopus* F2a2 moves slower than that of calf making it harder to resolve from F2b.

The F1 histones of *Xenopus* are complex. Acid–urea gels resolve three F1-like proteins from cultured cells (Figure 1) while only two are apparent on sodium dodecyl sulfate gels. Histones from *Xenopus* nucleated erythrocytes (not shown) give several extra bands in the F1 region. As we will show later, at least one of these "extra" bands is also present in cultured cell histones for it becomes strongly iodinated under certain conditions. It is not clear which one of these F1 histones, if any, should be designated F2c, similar to the serine, lysine-rich histone found in avian erythrocytes (Edwards and Hnilica, 1968; Nelson and Yunis, 1969).

Comparison of Iodinated Peptides from Calf Thymus and Xenopus Histones. Calf and *Xenopus* histones were iodinated in the presence of 5 M urea, 2 M NaCl and electrophoresed on acid–urea gels. After cutting the gels into 1-mm slices, the protein was extracted from each slice and digested with trypsin, and the peptides were separated by electrophoresis at pH 3.5. The ^{125}I-labeled peptides were then located by autoradiography.

The results of such analysis are shown in Figure 3. All the major histones become iodinated in 5 M urea–2 M NaCl. However, only F2a1, F2b, and some of the F1's were labeled heavily enough and/or resolved well enough to examine their iodinated peptides. For the F1 histones it was clear that the iodinated peptides differ considerably between the two species (data not shown). This merely confirms the already established fact that the F1 group changes the most rapidly during evolution (Panyim et al., 1971) and they were not examined further. The iodinated F2b peptides are quite similar between calf and *Xenopus*, suggesting similar amino acid sequences. However, calf F2b has only three tyrosine-containing tryptic peptides whereas we find at least five radioactive spots on the peptide map. The reason for

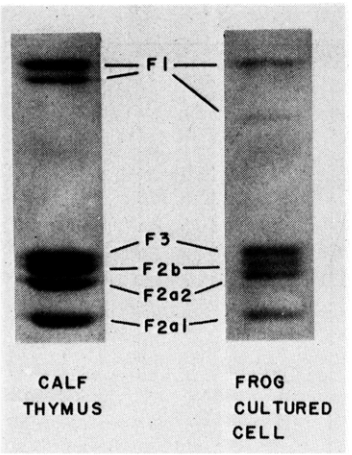

FIGURE 2: Sodium dodecyl sulfate gels of calf thymus and *Xenopus* histones. A 15% sodium dodecyl sulfate slab gel was prepared according to Laemmli (1970) except that bisacrylamide was lowered to 0.2%. About 10 μg each of the calf thymus or *Xenopus* cultured cell histones was heated to 100 °C in sodium dodecyl sulfate sample buffer and then applied to the slots in the stacking gel. The gel was electrophoresed at 60 V for 2 h and then at 120 V for 5–7 h and then was stained with Coomassie blue G-250. Electrophoresis was from top (−) to bottom (+).

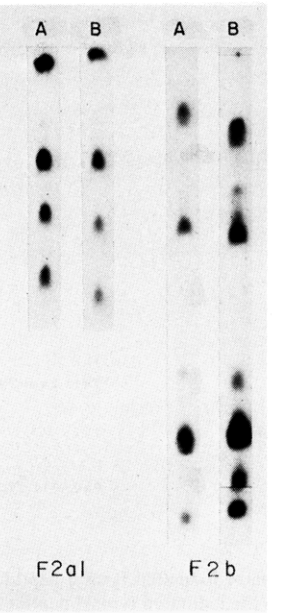

FIGURE 3: Iodinated peptides from *Xenopus* and calf thymus histone F2a1 and F2b. Acid-extracted and lyophilized histones from (A) *Xenopus* or (B) calf thymus chromatin iodinated in a denaturing solvent (5 M urea, 2 M NaCl) were dissolved in 10 M urea and electrophoresed on gels as described in Figure 1. The gels were sliced in 1-mm pieces, and the radioactivity in each piece was determined by γ counting. The radioactive histone was eluted from each 1-mm gel slice, lyophilized, digested exhaustively with trypsin, and subjected to high voltage paper electrophoresis at pH 3.5, 3000 V for 2 h. The tyrosine-containing tryptic peptides from each species are visualized by an overnight exposure to x-ray film and are shown side-by-side for comparison. *Xenopus* F2a1 shows four spots which co-migrate with those of calf thymus. More than the expected three spots are visible for F2b. The peptide maps of frog and calf F2b are similar but not identical.

this discrepancy is not known. We have not studied the F2b peptides any further.

Calf F2a1 is cleanly resolved from the other histones in the acid–urea gels and yields four iodinated tryptic peptides as expected from its known amino acid sequence (DeLange et al., 1969). *Xenopus* F2a1 behaves in all respects like its calf counterpart.

In summary, calf and *Xenopus* F2a1 cannot be distinguished on the basis of electrophoresis in acid–urea or sodium dodecyl sulfate gels, their extractability from chromatin, and the number and mobility of their iodinatable tryptic peptides. Considering that F2a1 shows only two, conservative amino acid differences between cows and peas (DeLange et al., 1969), it is reasonable to conclude that calf and *Xenopus* F2a1 are very similar, if not identical, in amino acid sequence. For this reason, coupled with its ease of isolation, we have focused on F2a1 in the experiments to be described hereafter.

Identification of the Iodinated Peptides in Calf Thymus F2a1. We next determined where each of the iodinated tryptic peptides was located in the overall amino acid sequence. Figure 4 gives the sequence of the four tyrosine-containing tryptic peptides and shows their location in the F2a1 molecule. F2a1 contains a single methionine located in the peptide containing tyrosyl 88. Cleavage at this methionine with cyanogen bromide (CNBr) should yield two large fragments, each containing two tyrosyls (DeLange et al., 1968). The smaller CNBr fragment upon tryptic digestion should yield the carboxy-terminal tyrosyl peptide (tyrosyl 98) and an altered tyrosyl 88 peptide due to the methionine cleavage. Figure 5 shows the result when iodinated F2a1 is cleaved with CNBr and the fragments separated by gel electrophoresis. The expected two fragments are seen plus a considerable amount of uncleaved F2a1. This low yield of cleavage products is most likely due to the fact that the oxidizing conditions required for iodination convert some of the methionine to a sulfoxide or sulfone which is resistant to CNBr (Gross, 1967). Noniodinated F2a1 was cleaved in good yield under these conditions.

The tryptic peptides from uncleaved F2a1 and from both CNBr fragments are also shown in Figure 5. The second fastest migrating tryptic peptide comes from the carboxy-terminal CNBr fragment and is unaltered by CNBr treatment. This fact identifies it as containing tyrosyl 98. The other tryptic peptide from the carboxy-terminal CNBr fragment has been altered by the CNBr cleavage and is thus identified as containing tyrosyl 88.

To distinguish which of the two peptides from the amino-terminal CNBr fragment contained tyrosyl 51 and 72, iodinated F2a1 was trypsin digested and electrophoresed at pH 3.5. The separated peptides were then electrophoresed in a second dimension at either pH 6.5 where acidic peptides migrate toward the positive electrode, or at pH 1.9 where peptides migrate according to the number of positive groups. The slowest migrating peptide moved to the positive electrode at pH 6.5, identifying it as the one containing tyrosyl 51 (and two glutamic acid residues), and the fastest migrating spot moved ahead of all other peptides at pH 1.9, identifying it as the one containing tyrosyl 72 (and a positively charged histidine residue).

In summary, the iodinated tyrosine peptides from calf thymus F2a1 were found to contain tyrosyl 51, 88, 98, and 72 going from slowest to fastest at pH 3.5. Since the iodinated peptides from *Xenopus* F2a1 cannot be distinguished from those of calf, we have used the calf sequence to order the *Xenopus* peptides also.

Iodination of Histones on Chromatin in Various Solvents. Reactivity toward iodine was examined in native chromatin (dissolved in 5 mM Tris, 1 mM EDTA) and also in chromatin which had been disrupted in the following sol-

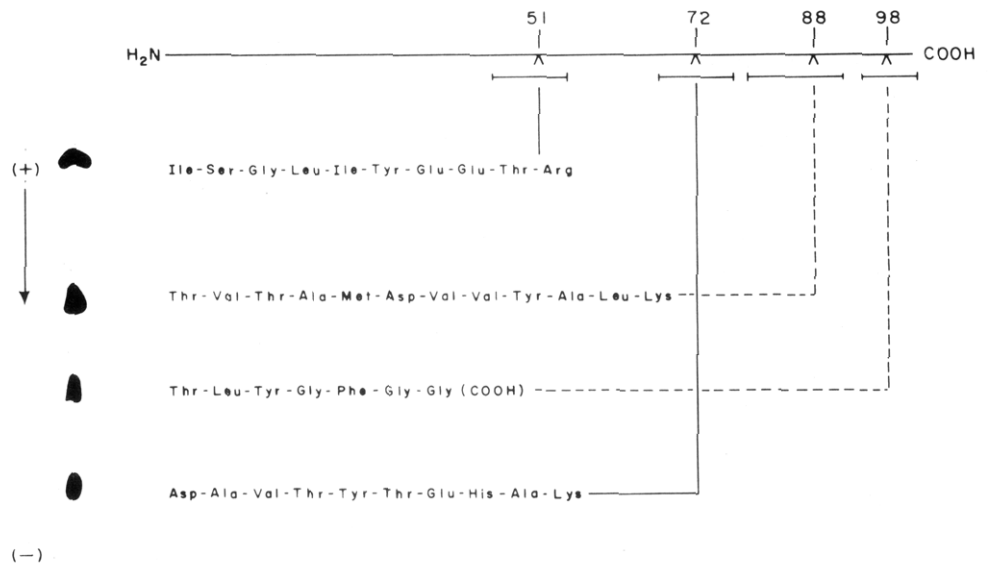

FIGURE 4: Location of iodinated tyrosyl peptides in *Xenopus* F2a1 histone. *Xenopus* F2a1 was iodinated, digested with trypsin, and electrophoresed at pH 3.5, and the iodinated tyrosyl peptides were located by autoradiography (left). The peptides were identified by a variety of procedures including electrophoresis at various pH's and cyanogen bromide cleavage (see text). Assignments were made assuming that the *Xenopus* peptides have the same amino acid sequence as those from calf thymus.

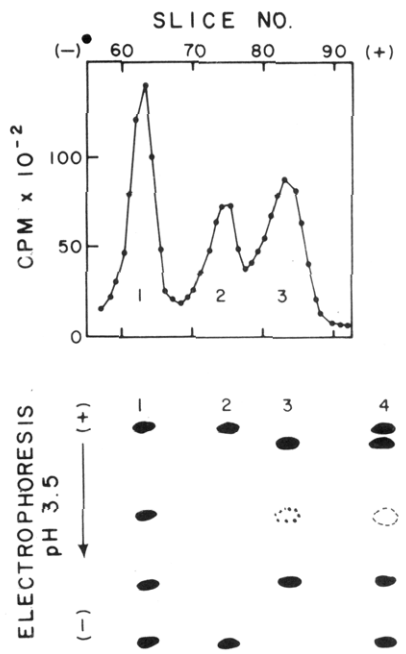

FIGURE 5: Cyanogen bromide cleavage of iodinated calf thymus F2a1. Purified calf thymus F2a1 was iodinated in 10 M urea, cleaved by cyanogen bromide in 70% formic acid, and electrophoresed on acrylamide gels, which were sliced and γ counted. The fastest moving band is the 2000 dalton carboxy-terminal fragment (3), the center band is the 8000 dalton amino-terminal fragment (2), and the slowest moving band is uncleaved F2a1 (2). Electrophoresis was from left (+) to right (−). Each fragment was eluted from the gel, digested with trypsin, and electrophoresed at pH 3.5. Lane 1, uncleaved F2a1; lane 2, fragment 2; lane 3, fragment 3; and lane 4, CNBr-treated F2a1 (about 70% cleaved).

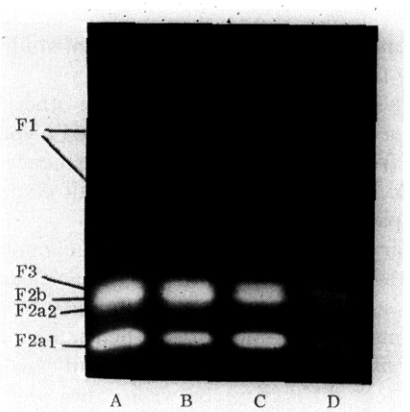

FIGURE 6: Autoradiograph of sodium dodecyl sulfate gel of histones iodinated on chromatin dissolved in various solvents. *Xenopus* cultured cell chromatin was dissolved in (A) 2 M NaCl, 5 M urea, (B) 5 M urea, (C) 2 M NaCl, or (D) 5 mM Tris, 1 mM EDTA and iodinated, and the histones were extracted in 0.4 N sulfuric acid. An equivalent amount of histone from each of the four samples was lyophilized, dissolved in sodium dodecyl sulfate sample buffer along with 10 μg of marker *Xenopus* histones, heated to 100 °C for 1 min, and electrophoresed in sodium dodecyl sulfate slab gel as described in Figure 2. The slab gel was stained, dried, and autoradiographed. Histones from denatured chromatin contain more radioactivity than those from native chromatin. F2a2 and F1 only became labeled when the chromatin was disrupted with NaCl, urea, or both.

vents: 2 M NaCl which removes histones from DNA but leaves the histones highly aggregated (Edwards and Shooter, 1969); 5 M urea which partially destroys tertiary structure but does not remove histone from DNA (Bartley and Chalkley, 1972); and 5 M urea plus 2 M NaCl which together removes histones from DNA and partially denatures internal protein structure. The reactivity of isolated F2a1 was also examined in 10 M urea, a solvent which destroys essentially all tertiary structure (Tanford, 1961). The initial aim was to determine which tyrosyls are protected by association with DNA (i.e., exposed by 2 M NaCl) and which are protected by association with other protein (i.e., exposed by 5 M urea).

Xenopus chromatin was adjusted to 1 mg/ml of Lowry-positive material (Lowry et al., 1951) and iodinated in each of the above solvents in the presence of 5 nmol of L-tyrosine. The tyrosine serves as a freely diffusible internal standard which we assume to be completely accessible to iodine and whose reactivity serves as a standard which can be compared with the reactivity of each peptide tyrosyl. After

stopping the reaction, each mixture was extracted with acid, the pellet spun out, and the supernatant was passed through a small column of Bio-Gel P-10. The void volume contained the iodinated histones, while the column volume contained unreacted iodine and iodinated tyrosine. A portion of the pooled included volume was rechromatographed on a Bio-Gel P-2 column for quantitative determination of the radioactivity associated with iodotyrosine in each separate iodination reaction.

Iodinated histones were electrophoresed on sodium dodecyl sulfate-acrylamide gels to determine which of the histones became iodinated. The results are shown in Figure 6. In native chromatin all the histones label to some extent except F2a2 which only becomes labeled as the chromatin is disrupted; F1 is only very slightly labeled. The radioactive F1 band co-migrates with the minor stained F1 band, and there is no significant radioactivity which co-migrates with the major staining F1 band.

Another portion of the radioactive histones was electrophoresed on acid-urea acrylamide gel to separate F2a1 from the other histones. The radioactive protein corresponding to the F2a1 region of the gel was eluted from the gel and digested with trypsin, and the tyrosine-containing tryptic peptides analyzed by electrophoresis at pH 3.5. The amount of radioactivity in each spot was determined and the micromoles of peptide estimated from the input amount of histone to obtain a specific radioactivity of the peptide.

Reactivity indices for each of the four tyrosine-containing peptides in F2a1 from chromatin iodinated under various solvent conditions are shown as a histogram in Figure 7. In native chromatin all of the F2a1 tyrosyls are accessible to iodine. However, they are considerably less reactive than free tyrosine, suggesting that the chromatin structure is partially protecting them. In addition, the tyrosyls are not equally reactive. Tyrosyl 51 and 88 account for most of the label. To our surprise, disrupting the chromatin with either 5 M urea, 2 M NaCl, or a combination of the two had almost the same effect on F2a1 iodination despite the known differences in their mode of action. In each case there was a five- to tenfold increase in the reactivity of all tyrosyls. We conclude that neither protein-protein interactions nor DNA-protein interactions are by themselves able to afford protection from iodination. Presumably the protection is afforded by the overall structure of the chromatin, and disruption of either aspect of it is enough to expose the tyrosyl residues.

For tyrosyl 72 and 98 another level of protection can be distinguished which appears to come from protein-protein interactions. Iodination of chromatin in 10 M urea (experiment not shown) or isolated F2a1 in 10 M urea has the same effect. The reactivity of tyrosyl 72 and 98 is increased so that all four tyrosyls have about the same reactivity.

Iodination of Chromatin in Increasing NaCl Concentrations. There was a large increase in the reactivity index of all tyrosyls when native chromatin was dissociated with 2 M NaCl. To determine at what NaCl concentration the increase in reactivity occurred, *Xenopus* chromatin was iodinated in increasing concentrations of NaCl. Since histones are removed in a stepwise fashion with increasing concentrations of NaCl, a stepwise increase in reactivity might be correlated with removal of one or more of the histones.

Xenopus chromatin was iodinated in the presence of L-tyrosine while dissolved in: (A) 5 mM Tris, 1 mM EDTA; (B) 0.3 M NaCl; (C) 0.5 M NaCl; (D) 0.75 M NaCl; (E) 1.0 M NaCl; (F) 2.0 M NaCl. F2a1 was then purified from each sample by gel electrophoresis and trypsin digested, the peptides were separated by electrophoresis at pH 3.5, and the amount of radioactivity in each peptide spot was determined. The reactivity index of each F2a1 tyrosyl was calculated and plotted as a function of NaCl concentration in Figure 8. Three of the F2a1 tyrosyls (98, 72, and 51) behave in a qualitatively similar fashion: their reactivities remain essentially unchanged until the NaCl concentration reaches 1 M. Between 1 M and 2 M these three undergo a five- to tenfold increase in reactivity. Tyrosyl 88, however, behaves in a quite different manner. It attains nearly maximum reactivity at 0.5 M NaCl and undergoes little change from there on.

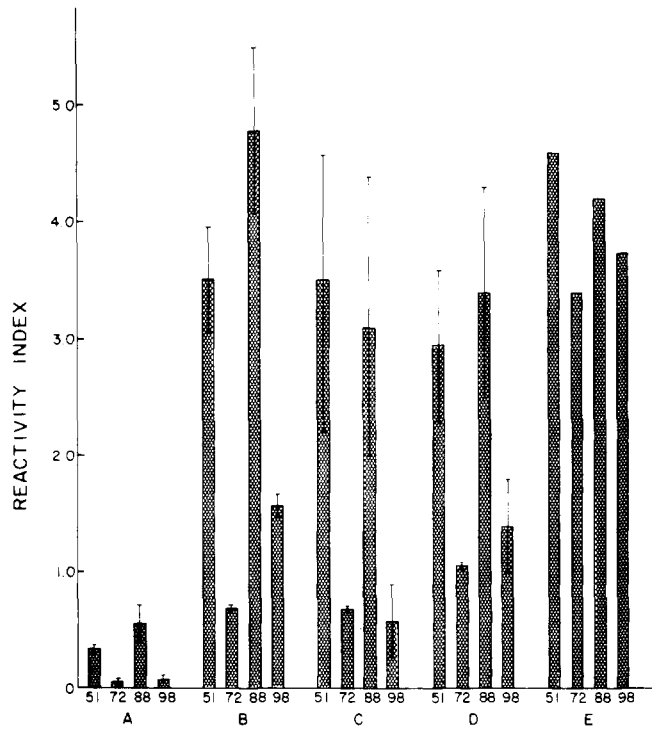

FIGURE 7: Histogram of reactivity indices. The histogram shows the reactivity indices of the four tyrosyls of F2a1 from *Xenopus* chromatin iodinated in (A) 5 mM Tris, 1 mM EDTA, (B) 2 M NaCl, (C) 5 M urea, (D) 2 M NaCl, 5 M urea and for calf thymus F2a1 iodinated in (E) 10 M urea. The error bars in A through D show the variation from two separate iodinations. The reactivity index was calculated from the specific activity of each peptide tyrosyl and the specific activity of L-tyrosine added to each iodination reaction mixture which served as a freely diffusible internal standard. Chromatin disruption caused a five- to tenfold increase in reactivity index. Isolated F2a1 denatured in 10 M urea iodinated each tyrosyl equally, but the peptide tyrosyls were about four times more reactive than free tyrosine.

Xenopus chromatin was examined to see which histones are removed at various NaCl concentrations. Chromatin was brought to the desired NaCl concentration, DNA and attached proteins were pelleted through a sucrose cushion, and both pellet and supernatant were examined for their histone content. At 0.3 M NaCl, chromatin was a precipitate and no histones were extracted. At 0.5 M NaCl, nearly all F1 was removed but the other histones were present in normal amounts. Between 0.75 M and 2 M NaCl the rest of the histones were progressively removed in concert. These results are similar to those of Ohlenbusch et al. (1967) who used calf thymus chromatin. The increase in reactivity of tyrosyl 88 correlates very well with the removal of F1. The increases in the other tyrosyls correlate with the gradual removal of all the other histones from the DNA. This experi-

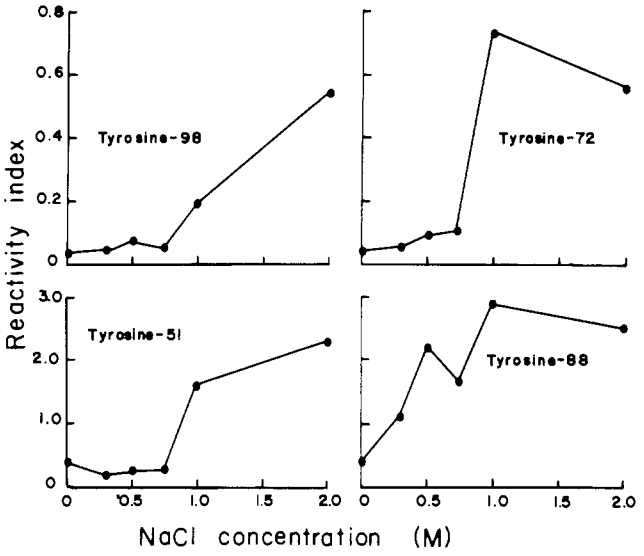

FIGURE 8: Reactivity of histone F2a1 tyrosyls iodinated in chromatin in increasing NaCl concentrations. *Xenopus* chromatin was dissolved in (1) 5 mM Tris, 1 mM EDTA, (2) 0.3 M NaCl, (3) 0.5 M NaCl, (4) 0.75 M NaCl, (5) 1.0 M NaCl, and (6) 2.0 M NaCl and then iodinated in the presence of an internal standard. The histones were extracted and electrophoresed on acid-urea gels. The radioactive F2a1 was eluted from the gel piece, trypsin digested, and electrophoresed at pH 3.5 to determine how much radioactivity was associated with each tyrosyl residue. The specific activity of each peptide tyrosyl was compared with the specific activity of free tyrosine for each respective NaCl concentration. Reactivity index is plotted as a function of NaCl concentration for each F2a1 tyrosyl.

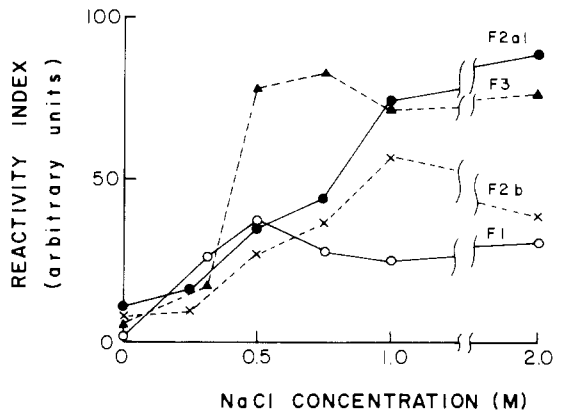

FIGURE 9: Reactivity of each histone as a function of NaCl concentration. *Xenopus* chromatin was dissolved in NaCl solutions as described in Figure 8 and iodinated in the presence of L-tyrosine. Equivalent amounts of histone from each chromatin preparation were loaded into separate slots of an sodium dodecyl sulfate slab stacking gel and electrophoresed and autoradiographed as described in Figure 6. The autoradiograph was traced with a densitometer and the area under each peak determined on a curve integrator. The relative amount of radioactivity of each histone (the area under the peak) was divided by the specific activity of tyrosine to calculate a relative reactivity index for each histone. F2a2 could not be quantitated on the densitometer but by visual inspection could be seen to increase gradually with increasing NaCl concentration. F3 (▲- - -▲) undergoes a large increase in reactivity at 0.5 M NaCl, whereas the other histones F2a1 (●- - -●), F2b (X- - -X), and F1 (O—O) appear to increase gradually with increasing NaCl concentrations.

ment suggests, therefore, that tyrosyl 88 may be involved in the interaction between F1 and F2a1.

Samples of histone from the chromatin iodinated in increasing NaCl concentrations were electrophoresed, the gels were dried and autoradiographed, and the radioactivity associated with each band was measured with a densitometer. The radioactivity associated with each histone was normalized to the specific activity of free tyrosine labeled in the same reaction to obtain a relative reactivity index for each histone. These reactivity indices are plotted as a function of NaCl concentration in Figure 9. Both F2a1 and F2b show a gradual increase in reactivity until they reach a maximum at 1 M NaCl. We know that for F2a1 this represents the sum of four tyrosyls, one of which achieves maximum reactivity at 0.5 M NaCl and three whose reactivities increase more gradually. This raises the possibility that, if F2b were analyzed in similar detail, one or more of its tyrosyls would also be found to reach maximum reactivity at 0.5 M NaCl. F2a2 was not resolved sufficiently to allow quantitation, but visual inspection suggested that it behaves similarly to F2a1 and F2b.

Histone F3 and the minor F1 band both reach maximum reactivity in 0.5 M NaCl. This suggests that most of the F3 tyrosyls (three in calf thymus) are uncovered by removal of F1.

Iodination of Free Tyrosine. The reactivity of each tyrosyl has been expressed as a ratio of its specific activity relative to that of free tyrosine in the same reaction. Such an approach is only valid when a small fraction of the available tyrosyls react before the iodine is used up. Iodine was limiting in all chromatin iodinations so the amount of iodine incorporated was a reflection of the initial reaction rate. Since all results have been normalized to the reactivity of free tyrosine, it is important to known how the various solvents affect the initial reaction rate of the free amino acid standard.

The rate of tyrosine iodination was measured and the results are shown in Table I. The half-time for iodotyrosine formation was 46 s with no added solvents but, in urea, NaCl, or both together, the rate decreased giving half-times of 59, 68, and 72 s. Thus, iodination of free tyrosine in salt or urea causes about a 1.5-fold depression in the reaction rate, although the reason for the depression is not clear. The slight difference in reaction rates does not invalidate any of our basic conclusions.

Discussion

When histone F2a1 is iodinated in native chromatin, all four tyrosyls are labeled but tyrosyl 88 and 51 have a reactivity about fourfold higher than the other two. Upon disruption of the chromatin with either 2 M NaCl or 5 M urea, all four tyrosyls show a five- to tenfold increase in reactivity, but their reactivities relative to each other remain about the same. Thus, from these results alone we cannot distinguish tyrosyls which are protected by interaction with DNA from those protected by protein interaction as was originally hoped. Most likely the protection observed in native chromatin is due to the overall folding of the nucleoprotein and disruption of this structure by either solvent unmasks all four tyrosyls. It is probably, however, that tyrosyl 72 and 98 are afforded additional protection solely by protein-protein interaction since raising the urea concentration to 10 M raises their reactivity to equal that of the other two.

Raising the NaCl concentration in increments showed that tyrosyl 88 attains maximal reactivity at 0.5 M NaCl. This correlates precisely with the selective removal of histone F1 from the chromatin. It suggests that tyrosyl 88 may be involved in F1 binding. However, alternate explanations are possible and it will require more experiments to decide among them. It is also intriguing to note that the F3 histone becomes maximally reactive in 0.5 M NaCl.

Table I: Rate of Tyrosine Iodination.[a]

Solvent	pH[b]	Final Absorbance 320 nm	No. of Trials	Time for Half-Maximal Absorbance (s)
(1) Tris buffer	8.10	0.160	8	46.2 ± 2.3
(2) 10 M urea	8.55	0.140	6	65.8 ± 3.2
(3) 5 M urea	8.30	0.160	5	59.0 ± 2.9
(4) 5 M urea, 2 M NaCl	8.27	0.190	5	68.0 ± 3.4
(5) 2 M NaCl	8.15	0.280	6	72.1 ± 12.1

[a] The rate of tyrosine iodination was measured in 1.0-ml volume containing the various solvents and 0.5 μmol of L-tyrosine, 0.12 μmol of KI, and 10 μmol of Tris buffer at pH 8.0. The increase in A_{310} was monitored at 10-s intervals after the addition of 0.14 μmol of Chloramine-T in 20 μl which initiated the reaction. [b] The pH of the reaction mixture was measured prior to addition of Chloramine-T.

So far we have assumed that the major factor governing the reactivity of tyrosyls in F2a1 is their topological availability to iodine. However, the microenvironment surrounding the tyrosyl may influence the pK_a of the phenolate moiety (Tanford, 1962) and thus may influence the iodination reaction (Hughes, 1957). In particular, neighboring groups may either raise or lower the pK_a. Basic groups tend to lower the pK_a (and increase reactivity to iodine), while acidic groups raise the pK_a (Perrin, 1965). We feel that the changes observed in reactivity of the F2a1 tyrosyls are due to steric factors rather than neighboring group effects for the following reasons. First, all four tyrosyls have nearly equal reactivity when the peptide chain is unfolded in 10 M urea. Second, three of the tyrosyls are surrounded (in the primary sequence at least) by aliphatic, apolar groups (see Figure 4). Only tyrosyl 51 is located next to a glutamic acid residue. If anything, this acidic neighbor should decrease the reactivity of tyrosyl 51. However, the results show that it is one of the most reactive even in native chromatin. Therefore, we feel that differences in reactivity are primarily due to topological availability of the tyrosyl residues. It is likely, however, that neighboring group effects are responsible for the observation that free tyrosine, with charged amino and carboxyl groups next to the reactive phenol ring, is fourfold less reactive than the tyrosyls in peptide linkage.

Previous investigators have used selective labeling agents on chromatin to investigate histone–histone interaction. Walker (1965) deduced that all tyrosyls and 20% of lysyl and arginyl residues of calf thymus chromatin were accessible to electrometric titration. We have concluded, however, that most tyrosyls are buried in native chromatin. The difference in results may lie in the fact that Walker (1965) used sonicated chromatin whereas we have prepared native chromatin by gentle lysis of nuclei. Simpson (1971), who also used sonicated chromatin, concluded that all tyrosyls were available for reaction with [^{3}H] acetic anhydride.

Hyde and Walker (1974) have assayed for buried or exposed sulfhydryl groups on histone F3 of calf thymus chromatin with DTNB. In native chromatin, only one of the two thiols reacted, but in isolated histone both are accessible. By treating chromatin with either NaCl or urea, they concluded that protection of the buried sulfhydryl is due to *inter*molecular interaction of F3 molecules.

The work of Weintraub and Van Lente (1974) corroborates our findings that 2 M NaCl and 5 M urea caused a five- to tenfold increase in accessibility to all tyrosyls and increasing the urea to 10 M made all tyrosyls equally accessible. Controlled trypsin digestion of native chromatin and chromatin in 2 M NaCl cleaves histones into discrete fragments (Weintraub and Van Lente, 1974), but histones in solution and on chromatin in 6 M urea are completely digested. The rate of digestion in 2 M NaCl was seven times faster, suggesting that the sites for trypsin digestion were more easily accessible while the specificity persisted.

Acknowledgments

We thank Drs. D. Fambrough, D. Powers, R. C. Huang, H. Weintraub, and R. Simpson for critical discussions of the data. We also thank Dr. D. Fambrough for the gift of purified calf thymus F2a1.

References

Bartley, J. A., and Chalkley, R. (1972), *J. Biol. Chem. 247,* 3647.

Bonner, J., Chalkley, G. R., Dahmus, M., Fambrough, D., Fujimura, F., Huang, R.-C. C., Huberman, J., Jensen, R., Marushige, K., Ohlenbusch, H., Olivera, B., and Widholm, J. K. (1968), *Methods Enzymol. 12B,* 3.

Brown, D. D., and Weber, C. S. (1968), *J. Mol. Biol. 34,* 661.

Chalkley, R., and Hunter, C. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1304.

D'Anna, J. A., and Isenberg, I. (1974), *Biochemistry 13,* 4992.

DeLange, R. J., Fambrough, D. M., Smith, E. L., and Bonner, J. (1968), *J. Biol. Chem. 243,* 5906.

DeLange, R. J., Fambrough, D. M., Smith, E. L., and Bonner, J. (1969), *J. Biol. Chem. 244,* 5669.

Diezel, W., Kopperschlager, G., and Hofmann, E. (1972), *Anal. Biochem. 48,* 617.

Edwards, L. J., and Hnilica, L. S. (1968), *Experientia 24,* 228.

Edwards, P. A., and Shooter, K. V. (1969), *Biochem. J. 114,* 227.

Fairbanks, G., Levinthal, C., and Reeder, R. H. (1965), *Biochem. Biophys. Res. Commun. 20,* 393.

Gross, E. (1967), *Methods Enzymol. 2,* 238.

Hamlin, J. L., and Arquilla, E. R. (1974), *J Biol. Chem. 249,* 21.

Hancock, R. (1974), *J. Mol. Biol. 86,* 649.

Herriot, R. M. (1947), *J. Gen. Physiol. 31,* 19.

Hewish, D. R., and Burgoyne, L. A. (1973), *Biochem. Biophys. Res. Commun. 52,* 504.

Hughes, W. L. (1957), *Ann. N.Y. Acad. Sci. 70,* 3.

Hunter, W. M., and Greenwood, F. C. (1962), *Nature (London) 194,* 495.

Hyde, J. E., and Walker, I. O. (1974), *Nucleic Acid Res. 1,* 203.

Johns, E. W. (1964), *Biochem. J. 92,* 55.

Kornberg, R. D., and Thomas, J. O. (1974), *Science 184,*

865.
Laemmli, U. K. (1970), *Nature (London) 227,* 680.
Litman, D. J., and Cantor, C. R. (1974), *Biochemistry 13,* 512.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Malchy, B., and Kaplan, H. (1974), *J. Mol. Biol. 82,* 537.
Martinson, H. G., and McCarthy, B. (1975), *Biochemistry 14,* 1073.
Nelson, R. D., and Yunis, J. J. (1969), *Exp. Cell Res. 57,* 311.
Noll, M. (1974), *Nature (London) 251,* 249.
Ohlenbusch, H. H., Olivera, B. M., Tuan, D., and Davidson, N. (1967), *J. Mol. Biol. 25,* 299.
Olins, A. L., and Olins, D. E. (1974), *Science 183,* 330.
Olins, D. E., and Wright, E. B. (1973), *J. Cell Biol. 59,* 304.
Panyim, S., Bilek, D., and Chalkley, R. (1971), *J. Biol. Chem. 246,* 4206.
Panyim, S., and Chalkley, R. (1969a), *Arch. Biochem. Biophys. 130,* 337.
Panyim, S., and Chalkley, R. (1969b), *Biochemistry 8,* 3972.
Perrin, D. D. (1965), Dissociation Constants of Organic Bases in Aqueous Solution, London, Butterworths.
Sahasrabuddhe, C. G., and Van Holde, K. E. (1974), *J. Biol. Chem. 249,* 152.
Simpson, R. T. (1971), *Biochemistry 10,* 4466.
Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N.Y., Wiley.
Tanford, C. (1962), *Adv. Protein Chem. 17,* 69.
Van Lente, R., Jackson, J. F., and Weintraub, H. (1975), *Cell 5,* 45.
Walker, I. O. (1965), *J. Mol. Biol. 14,* 381.
Weintraub, H., and Van Lente, F. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4249.

The Effect of Carboxymethylating a Single Methionine Residue on the Subunit Interactions of Glycophorin A†

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ABSTRACT: Human red cell glycophorin A shows an equilibrium between dimeric and monomeric forms which have been designated PAS-1 and PAS-2, respectively. This equilibrium, which is dependent upon protein concentration, is achieved by incubation in sodium dodecyl sulfate solutions at elevated temperatures and is assayed by sodium dodecyl sulfate gel electrophoresis. Carboxymethylation of glycophorin A in guanidine hydrochloride or urea alters the interactions between polypeptide chains so that the lower molecular weight form (PAS-2) is obtained much more readily. If the carboxymethylation is performed at pH 3.0, the reaction is limited to the two methionine residues of glycophorin A which are located at positions 8 and 81 in the sequence. In the presence of sodium dodecyl sulfate, only one of the two methionine residues is carboxymethylated, and glycoprotein modified under these conditions does not exhibit the change in electrophoretic mobility. Experiments with [1-^{14}C]iodoacetic acid demonstrated that Met-81, located in the hydrophobic domain of the protein, is the residue protected by sodium dodecyl sulfate. Modification of Met-81 destabilizes the dimeric form relative to the monomer by weakening the interactions between polypeptide chains. The experiments described in this paper confirm that the hydrophobic domain of glycophorin A is involved in subunit interactions and that Met-81 plays a critical role in those interactions.

The sialoglycoproteins of human erythrocyte membranes migrate as three PAS-positive bands when either ghost membranes (Fairbanks et al., 1971) or partially purified preparations (Furthmayr et al., 1975) are analyzed by sodium dodecyl sulfate gel electrophoresis. Recent studies from several laboratories indicate that two of these electrophoretic forms (PAS-1 and PAS-2) are interconvertible (Marton and Garvin, 1973; Tuech and Morrison, 1974; Furthmayr and Marchesi, 1976). This conclusion was based on experiments in which membranes or glycoprotein preparations were dissolved in sodium dodecyl sulfate solutions and heated prior to electrophoresis. After heating there is a shift in material from PAS-1, which is the major band in unheated preparations, to the position of the band of lower apparent molecular weight, PAS-2. A similar shift can be observed by gel filtration. The decrease in apparent molecular weight was interpreted as the breakdown of an oligomer to a smaller subunit (Marton and Garvin, 1973; Ji and Ji, 1974; Garvin et al., 1975). The extent of the conversion of PAS-1 to PAS-2 induced by heating the glycoprotein in sodium dodecyl sulfate solutions is inversely dependent on the protein concentration and is reversible (Furthmayr and Marchesi, 1976). These observations appear to exclude other explanations of the band shift such as peptide bond cleavage or conformational changes. The experiments described in this paper demonstrate that the same change in electrophoretic mobility, and presumably therefore the same dissociation of subunits, can be induced by chemical modification. It is further shown that a single residue of methionine is the critical site of the modification and that conversion of this methionine to a sulfonium salt destabilizes the dimeric form of gly-

† From the Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06510. *Received October 28, 1975.* This work was supported by a grant from the American Cancer Society BC-102B and Membrane Center Grant No. P17-GM 21714.