Changes in the Properties of Myosin Associated with Muscle Development*

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ABSTRACT: In the absence of a thiol reagent the ATPase activity of chick embryo skeletal muscle myosin is extremely unstable. However, no significant differences are observed in the ATPase activity during development. The loss of activity in the absence of thiol protection is greater for myosin at earlier stages of development and greater for white than for red muscle myosin at all ages. Oxidation of sulfhydryl groups is shown to parallel the loss of ATPase activity. Full activity can be regenerated by treatment with a thiol reagent. In the adult there is also a progressive loss of SH groups. The rate of oxidation is much less than for the embryo and is not associated with any significant loss of ATPase activity. The stability difference between embryonic and adult myosin may be a reflection of conformational differences between the two forms.

This possibility is lent further support by the demonstration that the interaction of embryonic myosin with actin leads to a stabilization of ATPase activity.

The molecular weight and subunit structure of myosin from mammalian striated muscle have been well defined (Gershman *et al.*, 1969). The protein has a molecular weight of 470,000, and is composed of an elongated helical core of two heavy chains (mol wt 215,000) and two light chains (mol wt 20,000). Expression of the biological activity of myosin depends on the interaction between the light and heavy chains (Stracher, 1969; Dreizen and Gershman, 1970).

With this understanding of the molecular structure of myosin it becomes more meaningful to undertake a comparative study of myosins from various species and from other muscle types. Differences in the specific ATPase activity of myosin from various species have been observed (Bailey, 1942; Perry, 1960). The enzymatic characteristics of cardiac and skeletal muscle myosins from the same species have been shown to differ (Brahms and Kay, 1963; Mueller, 1964), as do those of red and white muscle myosins from the same species (Barany *et al.*, 1965; Sreter *et al.*, 1966; Maddox and Perry, 1966). There has been a lack of agreement about the enzymatic properties of foetal myosin, and in particular about whether changes in enzymatic activity occur during the development of foetal muscle. Trayer and Perry (1966) observed increases in specific ATPase activity during development, and suggested that embryonic myosin was chemically different from adult myosin. On the other hand, Baril *et al.* (1967) found no differences in specific ATPase activity during development if polyribonucleotide contaminants were removed from myosin by treatment with ribonuclease.

The following investigation was initiated in an attempt to resolve these differences as well as to further characterize the myosin of embryonic skeletal muscle.

Materials and Methods

Crystalline ATP, β-diphosphopyridine nucleotide, phosphoenolpyruvate, and the enzymes pyruvic kinase and lactic dehydrogenase, in crystalline suspensions, were obtained from Sigma Chemical Co. Cleland's reagent (dithiothreitol) was obtained from Calbiochem Inc., Ellman's reagent (DTNB)[1] from Aldrich Chemical Co., and iodoacetamide from Mann Research Laboratories. Ribonuclease A was bought from Worthington Biochemical Corp., and Sephadex G-200 and DEAE A-50 were obtained from Pharmacia Fine Chemicals.

Purification of Myosin. Adult chicken myosin was prepared by procedures described previously (Dreizen *et al.*, 1966) with the addition of a final chromatographic fractionation on DEAE A-50 Sephadex, using the conditions outlined below for the purification of embryonic myosin. White muscle was obtained from the breast and red muscle from the legs.

The purification of chick embryo myosin required a more extensive procedure. Breast muscle was used as a source of predominately white muscle fibers and leg muscle as a source of predominately red muscle fibers. While it was recognized that chick embryo leg muscle is made up of red, white, and intermediate fibers, the high content of red fibers permits a valid comparison to be made between this sample and the almost exclusively white fibers of the breast muscle preparation. The infinitesmally small quantity of pure red muscle in the chick embryo made its use impractical.

All procedures were carried out at 4° unless otherwise specified. Muscle tissue was dissected from the breast and legs of 36 chick embryos and homogenized briefly in a Waring Blender. The homogenate was extracted for 15 min with a solution containing 0.4 M KCl, 0.05 M phosphate, 0.01 M pyrophosphate, and 0.005 M $MgCl_2$ at pH 6.5. After centrifugation the supernatant was treated with ribonuclease A (100 μg/ml) at room temperature for 5 min and then at 4° for 3–4 hr. This procedure was found to be essential for the removal of contaminating ribosomal material. Subsequent to the RNase treatment DTT was added to a final concentra-

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[1] Abbreviations used are: DTT, dithiothreitol (Cleland's reagent); DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent); IAA, iodoacetamide.

tion of 1 mM and was maintained at this level throughout the purification.

The extract was then dialyzed against 0.01 M phosphate–1 mM DTT at pH 7.0 for 2 hr. The precipitated myosin was collected, redissolved in an equal volume of 1.0 M KCl, 0.1 M phosphate, 0.01 M pyrophosphate, 0.005 M $MgCl_2$, and 1 mM DTT at pH 7.6, and centrifuged at 55,000*g* for 2 hr. The resulting supernatant was fractionated using a saturated $(NH_4)_2SO_4$ solution containing 10 mM EDTA and 1 mM DTT at pH 7.0. The fraction precipitating between 35 and 50% $(NH_4)_2SO_4$ saturation was collected, redissolved, and dialyzed against a 0.30 M KCl, 0.05 M phosphate, and 1 mM DTT solution at pH 7.6. This sample was subsequently chromatographed on a DEAE A-50 Sephadex column. Column dimensions were 60 × 1.5 cm and a continuous KCl gradient of 0.30–1.0 M KCl in 0.05 M phosphate–1 mM DTT (pH 7.6) was used as the eluting buffer.

Ca^{2+}ATPase activity was found to be associated exclusively with the first peak eluted, and this material had a 280/260 mμ of 1.3–1.5. Later fractions were contaminated with nucleotide material and had 280/260 mμ ratios less than 1.0.

The first component was precipitated by dialysis against 0.01 M phosphate–1 mM DTT (pH 7.0). The precipitate was redissolved in an equal volume of 0.8 M KCl–0.10 M phosphate at pH 7.6 and chromatographed on a Sephadex G-200 column using 0.4 M KCl–0.05 M phosphate–1 mM DTT (pH 7.6) as the eluting buffer. Column dimensions were 30 × 1.5 cm. Ca^{2+}ATPase activity was again associated exclusively with the first component eluted and this material had a 280/260 mμ ratio was 1.7–1.8. A total of 6–8 mg of purified breast muscle myosin and 15–20 mg of leg muscle myosin was obtained from 36 16-day embryos.

Preparation of Actomyosin. Natural actomyosin was extracted as described by Perry and Corsi (1958). Natural actomyosin was converted to desensitized actomyosin as described by Schaub *et al.* (1967). Removal of troponin and tropomyosin was indicated by the loss of the EGTA sensitivity of the Mg^{2+}-activated ATPase activity. Desensitized actomyosin was converted to myosin by centrifuging it at 150,000*g* for 3 hr in a solution containing 0.4 M KCl, 0.05 M phosphate, 0.005 M ATP, and 0.005 M $MgCl_2$ at pH 7.6 and 4°.

ATPase Activity. Ca^{2+}ATPase activity of myosin was measured by incubating myosin at 37° with 0.5 M KCl, 2.5 mM ATP, 2.5 mM $CaCl_2$, and 25 mM Tris-HCl at pH 7.6. The ADP produced was measured by coupling it to the oxidation of DPNH in the presence of phosphoenolpyruvate, pyruvic kinase, and lactic dehydrogenase at pH 7.6. The oxidation of DPNH was determined from the reduction in absorbance at 340 mμ in a Beckman DU spectrophotometer. Mg^{2+}-activated Ca^{2+}ATPase was determined by incubating the sample at 37° in a solution containing 2.5 mM ATP, 2.5 mM $MgCl_2$, 0.02 M Tris-HCl, and 0.01 mM $CaCl_2$, at pH 7.6. EGTA sensitivity was determined by incubating the sample with a solution containing 2.5 mM ATP, 2.5 mM $MgCl_2$, 1 mM EGTA, and 0.02 M Tris-HCl at pH 7.6. ADP released was measured as described above.

Protein Concentrations. Protein concentration was determined by optical density measurements at 280 mμ in a Beckman DU spectrophotometer with correction for Rayleigh scattering. $E^{1\%}_{280\,m\mu}$ was determined as 5.60 by Kjeldahl nitrogen determination.

Titration of Myosin with Iodoacetamide. Myosin (1–2 mg/ml) at pH 8.3 was incubated with 1.0, 2.5, 5.0, or 10.0 mM IAA at 25°. At various time intervals from 0.5 to 30 min, aliquots were removed and the reaction was terminated by the addition of a 5-fold volume of 0.4 M KCl–0.05 M phosphate containing a 5-fold molar excess of DTT over the concentration of IAA. The ATPase activity of each aliquot was determined.

FIGURE 1: Sedimentation velocity of purified myosin from breast muscle of 16-day chick embryo. Myosin in 0.4 M KCl–0.05 M phosphate, pH 7, 4°, 56,000 rpm. Scan taken 90 min after reaching speed.

Sulfhydryl Group Analysis. Free sulfhydryl groups were determined using Ellman's reagent (DTNB) (Ellman, 1959). Thiol reagent was removed from the sample by dialysis under N_2 immediately prior to the analysis. Myosin (200–500 μg) was treated with 5 M guanidine hydrochloride (pH 8.0) in a total volume of 1 ml for 10 min at room temperature. 50 μl of a solution of 10 mg/ml of DTNB was then added and the absorbance at 412 mμ was determined.

Sedimentation velocity studies were performed in a Beckman Model E analytical ultracentrifuge equipped with automatic temperature control, electronic speed control, and absorption optic system. Measurements were made at 280 mμ in a double-sector cell with a path length of 12 mm. Rotor speeds of 50,000–60,000 rpm were used at a temperature of 4°.

Results

Purification of Myosin. Inclusion of the final chromatographic procedure in the preparation of myosin from adult skeletal muscle, resulted in an increase of the 280/260 mμ ratio from 1.70 to 1.85 without significant change in the specific activity.

Purification of myosin from embryonic muscle by previously published methods (Trayer and Perry, 1966; Baril *et al.*, 1966) did not, in our hands, result in a preparation of acceptable purity. Sedimentation velocity studies in the analytical ultracentrifuge revealed that these preparations contained considerable quantities of slowly sedimenting material. Only with the development of the method described in the previous section were we able to obtain a preparation which sedimented as a single boundary with a sedimentation rate of 6.10 S (Figure 1). This preparation had a specific ATPase activity of 0.45–0.50 μmole/mg per min at pH 7.6 and a 280/260 mμ ratio of 1.70–1.80 after correction for Rayleigh scattering. ATPase activity was not activated by Mg^{2+} indicating the absence of actin contamination. Rechromatography of the purified sample on DEAE A-50 Sephadex resulted in the elution of myosin as a single symmetrical peak and the specific ATPase activity and 280/260 mμ ratio were unaffected.

Oxidation of Sulfhydryl Groups. Embryonic myosin prepared without the addition of DTT was found to have a markedly reduced specific ATPase activity. When DTT was present throughout the purification specific activities of 0.45–0.50 μmole/mg per min were obtained, while in the absence of DTT the specific activity was only 0.15–0.20. Activities within these limits were obtained from 40 separate preparations in the former case with at least 3 preparations at each age studied, and from 10 separate preparations in the latter. Each preparation was from 36 chick embryos of the same age varying

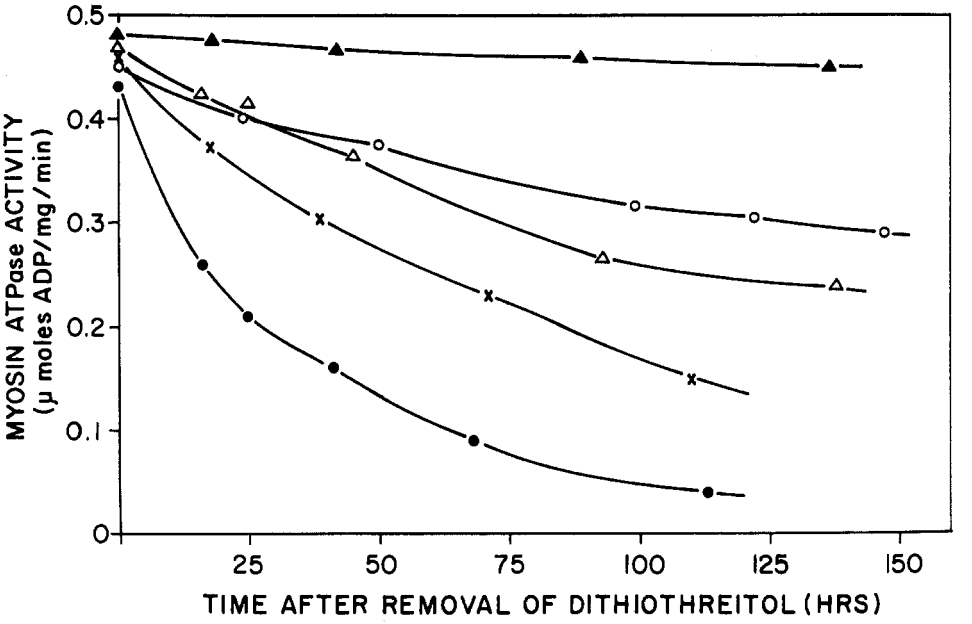

FIGURE 2: Effect of the removal of DTT on the ATPase activity of myosin. (●—●) 14-day embryo; (x—x) 16-day embryo; (△—△) 18-day embryo; (○—○) 20-day embryo; (▲—▲) 3-day hatched chick. DTT was removed by dialysis for 15 hr, and zero time was taken as the time at which dialysis was begun. Data from leg muscle myosin.

between the 13th and 20th days of incubation. Further investigation showed that although breast and leg muscle preparations retained comparable activities if prepared with DTT, the breast muscle myosin had a consistently lower specific activity than the leg muscle myosin when the purification was carried out in the absence of DTT. When DTT was removed from purified myosin samples, a rapid loss of activity was observed. The rate of loss of activity was however, greater for myosin prepared from younger embryos. A progressive increase in stability was apparent during development (Figure 2). In Figure 3 logarithmic rate constants for the loss of ATPase activity after the removal of DTT, are presented as a function of chick embryo age. These data indicate that, at all ages the loss of activity is greater for the breast muscle than for the leg muscle myosin preparations. Subsequent addition of DTT restored the specific activity to its previous level; the rate at which activity was restored being a function of the concentration of DTT added.

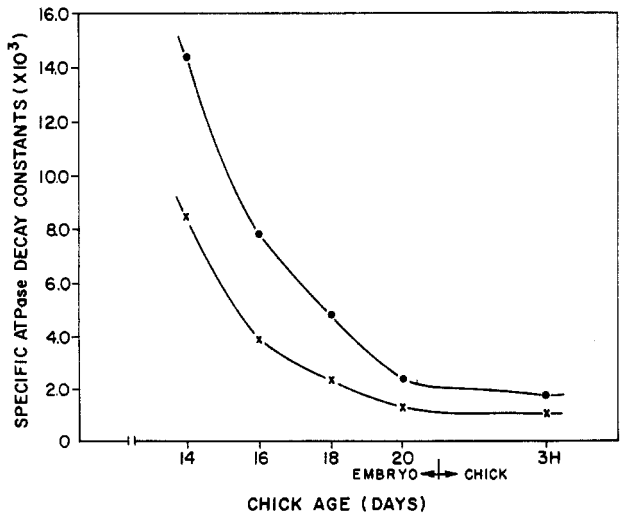

FIGURE 3: Rate constants for the loss of ATPase activity after removal of DTT. (●—●) Breast muscle myosin; (X—X) leg muscle myosin. Logarithmic decay constants, in arbitrary units, measure the loss of specific ATPase activity as a function of time after the removal of DTT. These rate constants were determined several times at each point and did not vary significantly.

Since these observations clearly imply that embryonic myosin contains a readily oxidizable group (or groups) which are critical to the maintenance of ATPase activity, an attempt was made to correlate the loss of ATPase activity with the number of free sulfhydryl groups. In 16-day embryo preparations, of both breast and leg myosins, the loss of 50–60% of the ATPase activity was associated with the loss of approximately the same percentage of the total number of free sulfhydryl groups (Figure 4).

In the absence of DTT the ATPase activity of embryonic myosin was partially protected by the addition of 1 mM EDTA. DTNB analysis demonstrated that there was again a good correlation between the loss of ATPase activity and the number of oxidized sulfhydryl groups (Figure 4). However, the addition of EDTA to partially inactivated myosin did not result in any restoration of the ATPase activity.

The effect of actin and the regulatory proteins troponin and tropomyosin was also investigated. Natural actomyosin was found to be stable in the absence of DTT; no significant loss of ATPase activity was observed over a period of 5 days in the absence of DTT. Natural actomyosin was then converted to desensitized actomyosin and again, in the absence of DTT, no loss of activity was observed over a 5-day period. Both the Ca^{2+}- and Mg^{2+}-activated ATPase activities of desensitized actomyosin were measured. Finally ATP was used to dissociate the actin–myosin complex, and immediately after removal of DTT from the myosin component, a rapid loss of Ca^{2+}-activated ATPase activity was observed.

Adult chicken red and white muscle myosin can be stored at 4° in the absence of DTT for several weeks, without loss of ATPase activity, and the addition of DTT produces no increase in the specific activity. However, under these conditions, adult myosin is subject to the loss of free sulfhydryl groups.

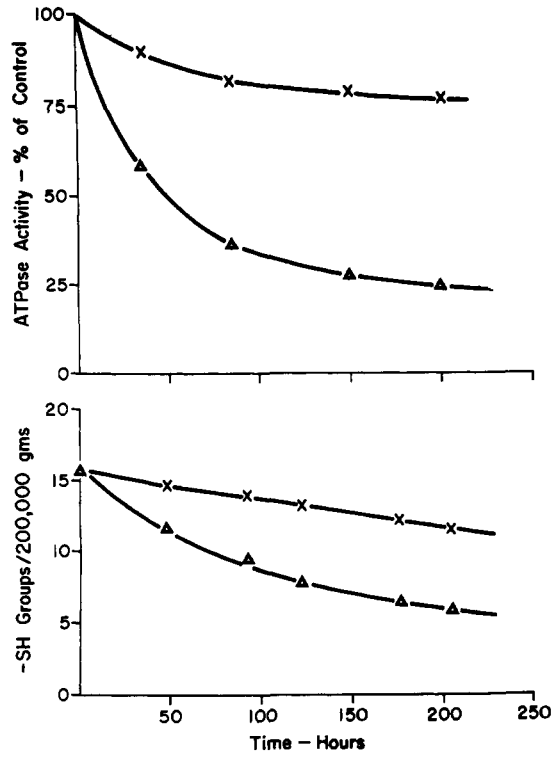

FIGURE 4: Loss of ATPase activity and SH groups after removal of DTT. (x—x) In the presence of EDTA; (Δ—Δ) in the absence of EDTA. Upper: loss of specific ATPase activity; lower: loss of free SH groups, as a function of time after the removal of DTT. Data from leg muscle myosin of 16-day embryos.

DTNB analysis showed that over a period of 28 days the number of free sulfhydryl groups dropped from 17.5 to about 2/200,000 g of myosin. No further loss of sulfhydryl groups was observed in the subsequent 6 days (Figure 5). The significance of these two remaining groups, and of maintaining them in the reduced state, was demonstrated by the subsequent attempt to label them with iodoacetamide. As the pH of this myosin solution was increased from 7.6 to 8.3, which presumably would have resulted in the ionization of the remaining sulfhydryl groups, there was a simultaneous and total loss of ATPase activity.

The effect of EDTA on the sulfhydryl groups of adult myosin was comparable to that observed for the embryonic samples; EDTA provided partial protection against oxidation of the sulfhydryl groups (Figure 5).

IAA Activation. In adult myosin samples iodoacetamide produced a maximal activation of ATPase activity approximately 8-fold greater than the control (Figure 6a). Maximal activation of embryonic myosin was only 4–6-fold greater than the control (Figure 6b). In neither case was any significant difference observed between red and white muscle myosins. Adult myosin was maximally activated within 60-sec incubation with 1.0 mM IAA while maximal activation of embryonic myosin within the same time period required incubation with 2.5 mM IAA. The normal biphasic response was seen in the adult within 20-min treatment with 5.0 mM IAA, while in the case of embryonic myosin 10.0 mM IAA was needed to produce a comparable degree of inhibition.

Sulfhydryl Groups. DTNB analysis revealed that myosin from adult red or white skeletal muscle has 17.5 ± 0.5 SH residues/200,000 g of myosin. Embryonic myosin, whether

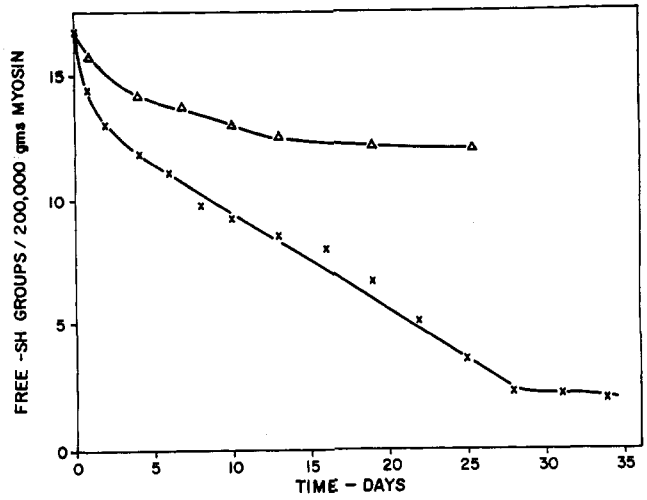

FIGURE 5: Oxidation of SH groups of adult myosin. (Δ—Δ) In the presence of EDTA; (x—x) in the absence of EDTA. Data are from myosin of chicken white muscle.

from breast or leg muscle, has only 15.5 ± 0.5 SH residues/200,000 g of myosin.

Discussion

Trayer and Perry (1966) reported that in early stages of embryonic muscle development the specific ATPase activity of myosin was only about 30% of that of adult muscle myosin. They suggested that these findings were a reflection of the replacement or modification of the foetal myosin by an adult type during development. Although they demonstrated a

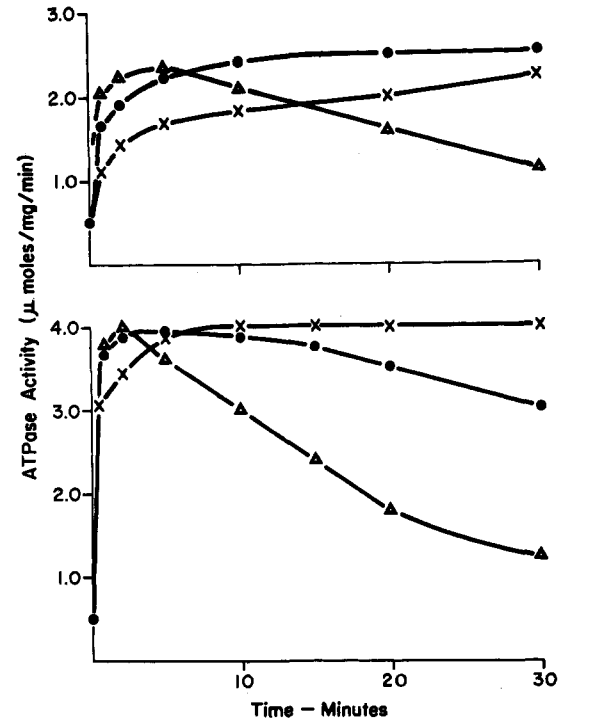

FIGURE 6: Titration of myosin with iodoacetamide. (a, bottom) Myosin from adult chicken. (x—x) 1.0 mM IAA; (●—●) 2.5 mM IAA; (Δ—Δ) 5.0 mM IAA. (b, top) Myosin from 16-day chick embryos. (x—x) 1.0 mM IAA; (●—●) 2.5 mM IAA; (Δ—Δ) 10 mM IAA.

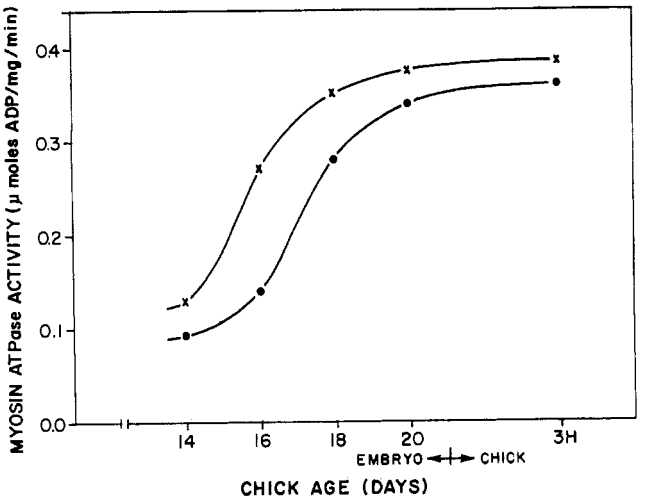

FIGURE 7: Residual ATPase activity 50 hr after removal of DTT. (x—x) Leg muscle myosin; (●—●) breast muscle myosin.

marked increase in specific activity on the addition of mercaptoethanol, it is unclear whether mercaptoethanol was routinely present in all their preparations. Baril *et al.* (1967) found no indication of age-dependent difference in specific activity.

The present study fails to indicate any significant differences in specific ATPase activity of myosin from chicks between the 14th day of incubation and 3 days after hatching. However, it does demonstrate that embryonic myosin is extremely susceptible to inactivation due to the oxidation of sulfhydryl groups. We also observed that, in the absence of DTT, myosin from less developed embryonic muscle was less stable, or more susceptible to oxidation, than that prepared from embryos at later stages of development. When ATPase activity was measured 50 hr subsequent to the removal of DTT from the purified sample (Figure 7), the relationship between residual activity and developmental age clearly was similar to that described by Trayer and Perry for their freshly prepared samples. However, their activities, of between 0.45 μmole/mg per min at 25° on the 12th day of incubation and 1.22 μmoles/mg per min 2 days after hatching, were considerably higher than those we have obtained. We are unable to explain the discrepancy but are not aware of any other reports of activities as high as these described by Trayer and Perry, even in preparations of adult muscle myosins. Specific activities reported by Trayer and Perry for foetal myosin chromatographed with 10 mM mercaptoethanol were even higher. This may in part be due to activation, by disulfide exchange with oxidized mercapthethanol, in their preparation.

Although EDTA provides partial protection against both oxidation of sulfhydryl groups and loss of ATPase activity, partially oxidized myosin can be reactivated only by the addition of a thiol reagent. It is interesting however, that embryonic actomyosin is not susceptible to loss of activity in the absence of DTT. Perhaps the interaction with actin stabilizes the conformation of the myosin molecule in such a way as to protect a critical sulfhydryl group or groups from oxidation.

Data from amino acid analysis do not suggest any notable changes in composition between embryonic and adult myosin, although sulfhydryl residues appear to be a little higher in the adult than in the embryo. Perry *et al.* (1970) have reported that the myosin from adult rabbit contains about two residues of 3-methylhistidine per mole of myosin while foetal myosin contains no methylated histidine residues. Although these differences may be significant with respect to the observed change in stability of developing myosin, it is still unclear whether they reflect a complete turnover of myosin between the embryonic and adult forms. Changes in the number or type of light chains during development probably would not be reflected in the amino acid composition of the whole myosin molecule.

Preliminary results from hybridization experiments, using the method described by Dreizen and Gershman (1970), indicate that the light chains from embryonic myosin can be recombined either with embryonic or adult heavy chains. Specific ATPase activities of the recombined myosin are comparable with those of the native form. Clearly any differences between embryonic and adult light chains do not affect those interactions between the light and heavy chains which are critical to the expression of ATPase activity. Adult light chains can be recombined equally effectively with embryonic or adult heavy chains.

Perry (1960) has suggested that adult and foetal myosin are chemically different and that this difference is expressed as an increase in specific ATPase activity during development. In the present study we did not observe any significant differences in ATPase activity during development of the chick embryo. However, this study does indicate a marked difference in the stability of embryonic and adult myosin. This difference is expressed in the embryo as a loss of ATPase activity associated with the oxidation of sulfhydryl groups. Both the rate of loss of ATPase activity and the oxidation of sulfhydryl groups is greater at earlier stages of development, and greater in white than red muscle myosin. Although adult myosin is susceptible to sulfhydryl oxidation, this is not associated with any loss of ATPase activity. The observation that all but 2 of the 17 SH residues can be oxidized without significant loss of ATPase activity raises interesting questions about the role of these 2 residues. This problem is presently under further investigation. Our observations lend support to the suggestion that adult and embryonic myosin may be chemically different. However this difference, rather than directly affecting the site of ATPase activity, probably produces conformational differences which secondarily affect the stability of sulfhydryl residue(s) which are critical for the expression of ATPase activity.

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Physical Properties of Human Follicle-Stimulating Hormone*

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ABSTRACT: The molecular weight of human follicle-stimulating hormone has been determined by both analytical ultracentrifugation at moderate concentrations and a combination of gel filtration and density gradient centrifugation at extremely low concentrations. The latter approach is by far the more discriminatory and useful for these studies. The results reveal that this glycoprotein hormone, as normally isolated from human pituitaries, is an extremely stable dimer of mol wt 35,000 ± 1000.

The hormone can be partially and irreversibly dissociated into its constitutive subunits by a variety of denaturing conditions (2–4 M guanidine hydrochloride, freezing and thawing) as well as prolonged standing in the cold in aqueous solution. At moderate concentrations, the latter process also results in the increased production of high molecular weight aggregates (*ca.* 500,000) of the glycoprotein.

The quantities of hormonal proteins of the pituitary which can be isolated as homogeneous preparations are normally small. In the past, this has prohibited extensive investigations of the molecular weight and other physical properties of these molecules. During recent years, however, advances in gel filtration chromatography as well as ultracentrifugation have made it possible to obtain an extensive amount of physical data on remarkably small quantities of material. With the advent of isolation procedures for homogeneous preparations of several of the gonadotropins (Squire *et al.*, 1962; Hartree *et al.*, 1964; Parlow *et al.*, 1965; Reichert and Jiang, 1965; Amir *et al.*, 1966; Roos, 1967; Saxena and Rathnam, 1967; Ryan, 1968; Peckham and Parlow, 1969) as well as convenient assay procedures (Faiman and Ryan, 1967a,b), we felt that an examination of the size, shape, and other physical characteristics of these proteins by the dual chromatographic and ultracentrifugal approach would be profitable. In this communication, we wish to report the results with one of these proteins, follicle-stimulating hormone (FSH),[1] isolated from human pituitaries.

Experimental Section

The preparations of human FSH employed in these studies are listed in Table I along with their biologic potencies and the methods of preparation. The biologic assays were performed using the rat ovarian weight augmentation assay for FSH (Steelman and Pohley, 1953) and the rat ovarian ascorbic acid depletion assay for human luteinizing hormone (Parlow, 1961) as previously described (Ryan, 1968). Radioimmunoassays were performed by the method of Faiman and Ryan (1967a,b).

Protein determinations were made either by obtaining the dry weight of newly lyophilized material or from solutions using the Lowry procedure (Lowry *et al.*, 1951). Protein determined by the Lowry method accounts for 76–80% of the dry weight of human FSH.

Radioiodination Procedure. Protein (1–2 μg) was labeled with ^{131}I by the method of Greenwood *et al.* (1963). Specific activities of 90–500 μCi/μg were obtained. ^{131}I-Labeled protein (2–5 ng) was applied to the gel filtration columns. ^{131}I activity was determined in an automatic gamma well counting system.

Treatment with Guanidine Hydrochloride. These experiments employed Ultra Pure guanidine hydrochloride, obtained from Mann Research Laboratories. The FSH was dissolved or diluted in the guanidine solutions at pH 6.5 and allowed to stand at room temperature for varying periods of time. The concentrations of guanidine hydrochloride and the incubation times are given in the Results section. Guanidine hydrochloride was not removed from the solutions prior to gel filtration, sedimentation, or assay but the concentration was lowered by dilution.

Analytical ultracentrifugation experiments were conducted with a Spinco Model E analytical ultracentrifuge equipped with Rayleigh and schlieren optics. Sedimentation velocity experiments with the standard proteins employed as calibrat-

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[1] Abbreviations used are: FSH, follicle-stimulating hormone; BSA, bovine serum albumin.