EVIDENCE FOR A NEW CARDIAC MYOSIN SPECIES IN THYROTOXIC RABBIT

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1. Introduction

Thyroid hormone may modulate the contractile behavior of the heart by influencing the ATPase activity of myosin. The velocity of myocardial contraction is increased in thyrotoxic animals [1–4] and the ATPase activity of myosin [5–8] and actomyosin are elevated [9,10]. In contrast, the ATPase activity of myosin is depressed after hypophysectomy and can be restored to normal by administration of thyroxine [11]. The mechanism whereby thyroxine exerts these effects is obscure, but a plausible explanation would be the induction of a myosin isozyme within the heart which has a higher ATPase activity. Recently, changes in myosin isozyme have been implicated in the modification of contraction velocities for skeletal muscle under different experimental conditions [12,13]. Further support of this concept is found in the observation that the rate of cardiac myosin replacement may be accelerated by thyroxine administration [14]. To date however, there has been no conclusive evidence that thyroxine induces a change in myosin structure.

Although Thyrum et al. [5] found changes in the amino acid composition and the helix content of cardiac myosin after thyroxine administration, subsequent investigations have failed to find abnormalities either in the amino acid composition or the electrophoretic mobility of the light and heavy subunits of cardiac myosin from thyrotoxic animals [6,8]. Also, the changes in ATPase activity induced by thyroxine apparently do not involve methylation of histidine residues or phosphorylation of myosin [8].

The techniques used by previous investigators could not have detected more subtle changes in myosin structure. In this paper we wish to report experiments demonstrating that thyroxine administration leads to the induction of a new cardiac myosin species. We have compared the electrophoretic mobility of CNBr digests of myosin-T with digests of myosin-N. These results show firstly that the electrophoretic pattern of the CNBr peptides of *S*-carboxymethylated myosin-T differs from myosin-N. Secondly, they demonstrate a difference in the distribution of radiolabeled cysteine containing peptides from these myosins. To our knowledge, this is the first definite evidence that thyroid hormone induces a new myosin species within the heart.

2. Methods

2.1. *Animal experiments*

Thyrotoxicosis was induced in three groups of seven young New Zealand white rabbits weighing about 1.0–2.0 kg by injecting L-thyroxine (200 μg/kg) daily for 14 days. Animals were weighed daily and the dose of thyroxine reduced to one-half, or omitted, if the body weight fell to less than 80% of the initial value. Uninjected rabbits from the same litters were used for control experiments.

2.2. *Myosin preparation*

Myosin was isolated from hearts as described earlier [8] and purified by chromatography on Sephadex A-50 according to the method of Richards et al. [15].

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Abbreviations: myosin-T, myosin isolated from thyrotoxic rabbit hearts; myosin-N, myosin isolated from normal rabbit hearts; SDS, sodium dodecyl sulfate

2.3. *Alkylation of myosin sulfhydryls*

The most rapidly reacting class of thiols, the so-called SH_1 thiols, in myosin were alkylated following the procedure described by Ohe et al. [16]. Myosin (2 mg/ml) in 0.5 M KCl, 0.05 M Tris–Cl, at pH 8.0, was reacted in an ice bath with a two-fold excess, on a molar basis, of iodo-[^{14}C] acetate (13.62 mCi/mM) or iodo-[^{3}H] acetate (207 mCi/mM). After 44 h the reaction was stopped by addition of a 100-fold excess of β-mercaptoethanol and the protein was denatured by adding dry urea to a final concentration of 8 M. This mixture was stirred under nitrogen for 20 h at 22°C to insure that all thiol groups were completely reduced. An amount of non-radioactive iodoacetate was added equivalent to 94% of the β-mercaptoethanol plus the number of cysteine residues in the sample. The reaction was allowed to proceed for 30 min in the dark. Glacial acetic acid was added to pH 4.0 to stop the reaction and the solution was dialyzed exhaustively against 25% acetic acid. These samples contained about 1.2–1.4 mol [^{3}H]- or [^{14}C]carboxymethyl cysteine/pmol. Values obtained from amino acid analysis varied between 38 mol and 42 mol carboxymethyl cysteine/mol, indicating that the cysteinyls had reacted almost quantitatively. To label all the thiol groups in myosin the protein was reduced and denatured as described above. Alkylation then was carried out using iodo-[^{14}C] acetic (5.10 mCi/mM) or iodo-[^{3}H] acetate (28.9 mCi/mM).

2.4. *Cyanogen bromide cleavage*

The dried protein was dissolved in 70% formic acid and treated for 17 h with a 200-fold excess, on a molar basis, of CNBr (Pierce Chemical). Amino acid analysis indicated a 92% loss of methionine.

2.5. *Gel electrophoresis*

CNBr digests of myosin were subjected to SDS-electrophoresis in 12.5% polyacrylamide gels containing 8 M urea and 0.1% SDS prepared and run essentially as described by Swank and Munkres [17]. For experiments in which the radioactivity in the gels was measured, the gels were prepared as described in the legend for fig.2. Actin (42 000 daltons), beef cardiac myosin light chain 1 (22 000 daltons) and the cyanogen bromide peptides of myoglobin [17] were run simultaneously as a guide to the approximate molecular mass of the peptides.

3. Results and discussion

The pattern obtained upon electrophoresis in 12.5% SDS–urea gels of CNBr digests of myosin-N and myosin-T are compared in fig.1. Digests of different preparations of myosin-N were very similar (fig.1(A,B)) and contained about 18 well-defined bands ranging in molecular mass from about 30 000 to about 3000 daltons. This number of bands is much smaller than the approximately 63 peptides which would be expected from the methionine content of cardiac myosin [6,8]. Although there were some additional larger peptides in the poorly-resolved material near the top of the gels, it is likely that many of the bands contained several peptides of similar molecular mass.

The patterns found with digests of myosin-T appeared similar to each other, but differed from normal in several respects (fig.1(C)). Thus digests of myosin-N contained a darkly staining band with an apparent molecular mass of about 15 000 daltons that was absent from preparations of myosin-T. Also, myosin-T contained darkly staining bands of about 22 000 daltons and 13 000 daltons, respectively, that were not observed in myosin-N. Also, there was a darkly staining band of about 26 000 daltons that corresponded to a lighter band in myosin-N.

Proteins are cleaved by CNBr rather specifically at the carboxyl terminus of methionine residues [18]. The alterations in the electrophoretic pattern described above indicate that myosin-T must contain methionine substitutions such that CNBr cleavage results in peptides with different mobilities than found normally. These substitutions probably occur in the heavy chains because the mass of the peptide involved is larger than any of those found in the light chains of cardiac myosin [19,20]. Additional studies using digests of separated myosin-T light chains will be needed to determine whether these subunits also are altered.

Blocking the rapidly reacting thiol groups in cardiac myosin usually leads to marked stimulation in Ca^{2+}-ATPase activity. By contrast, modification of the SH_1 thiol groups in myosin-T has no effect on Ca^{2+}-ATPase activity [6–8]. However, the SH_1 thiols in myosin-T seem to be readily accessible as the rate of incorporation of alkylating reagents is the same as in myosin-N [7]. These observations have led to the hypothesis that thyroxine may stimulate cardiac myosin ATPase

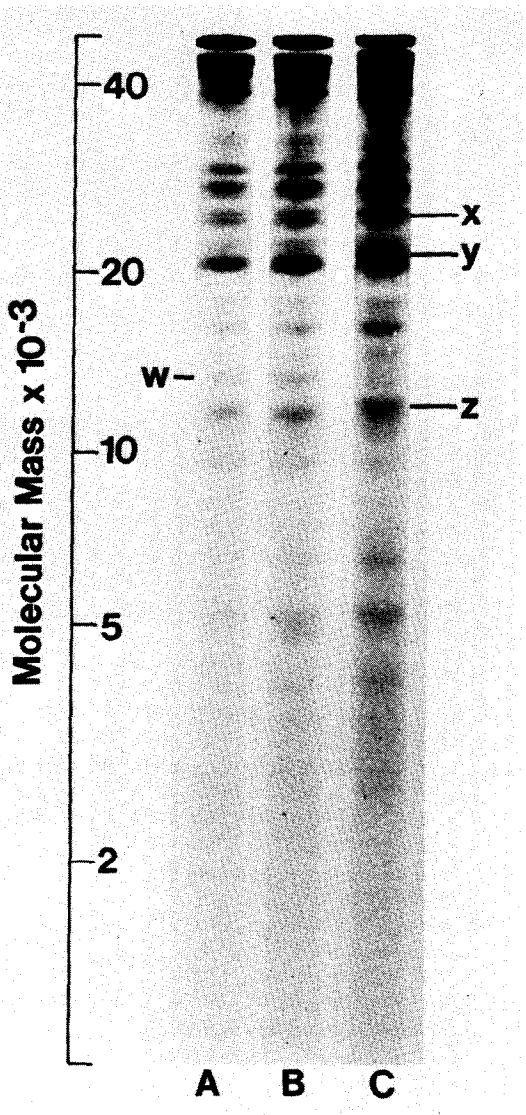

Fig.1. SDS–urea polyacrylamide gel electrophoresis of myosin CNBr digests. About 50 μg protein were applied to each gel. (A,B). Peptides from two preparations of myosin-N. (C) Peptides from myosin-T. Digests of myosin-N contained a band of about 15 000 daltons that was not found in digests of myosin-T (arrow w). Digests of myosin-T contained an intensely staining band of about 26 000 daltons that corresponded to a faint band in digests of myosin-N (arrow x) and bands in the 22 000 dalton and 13 000 dalton regions that were not present in digests of myosin-N (arrow y and z).

activity by a change in structure involving the region near the SH_1 thiols [6–8].

To identify the peptide(s) containing these thiol groups, CNBr digests of SH_1-labeled myosins were subjected to gel electrophoresis (fig.2). In myosin-N about 60% of the radioactivity was found in a peak which corresponded to a band in the gels with an apparent molecular mass of about 8500 daltons. A very similar pattern of labeling was obtained when myosin-T was reacted with iodo-[^{14}C] acetate under non-denaturing conditions (fig.2(B)). This result indicates that the alteration in banding patterns of myosin-T (fig.1) is not the result of a change in the mobility of the SH_1 thiol peptide.

Digests of myosins in which all the thiol groups had been labeled also were subjected to gel electrophoresis. The distribution of radioactivity obtained with digests of myosin-N (fig.2(A)) showed a peak in the 22 000 dalton region that was absent from digests of myosin-T (fig.2(B)). To exclude the possibility of variations in CNBr cleavage. [^{14}C]carboxymethylated myosin-T and [^{3}H]carboxymethylated myosin-N were mixed together before digestion with CNBr and then subjected

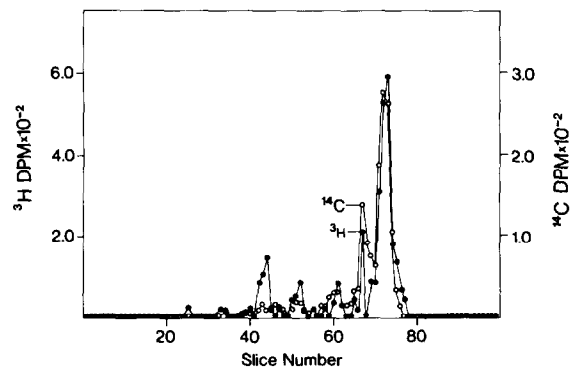

Fig.2. SDS-urea polyacrylamide gel electrophoresis of CNBr digests of myosins in which the SH_1 thiol groups were blocked using iodo-[^{3}H] acetate or iodo-[^{14}C] acetate. (A) Peptides from myosin-N. (B) Peptides from myosin-T. About 100 μg protein containing about 13 000 dpm ^{3}H or 3000 dpm ^{14}C were applied to 0.5 × 12 cm disk gels prepared as described in Methods, except that *N,N'*-diallytartardiamide was substituted for *N,N'*-methylene bisacrylamide [21]. Gels were frozen in dry ice, sliced at 1 mm intervals and dissolved by incubation in 0.7 ml 2% periodic acid for 1 h at 37°C. About 10 ml Aquasol (New England Nuclear) were added to each vial and the radioactivity was measured by liquid scintillation counting. Correction was made for quenching using [^{14}C]- and [^{3}H]-toluene as internal standards.

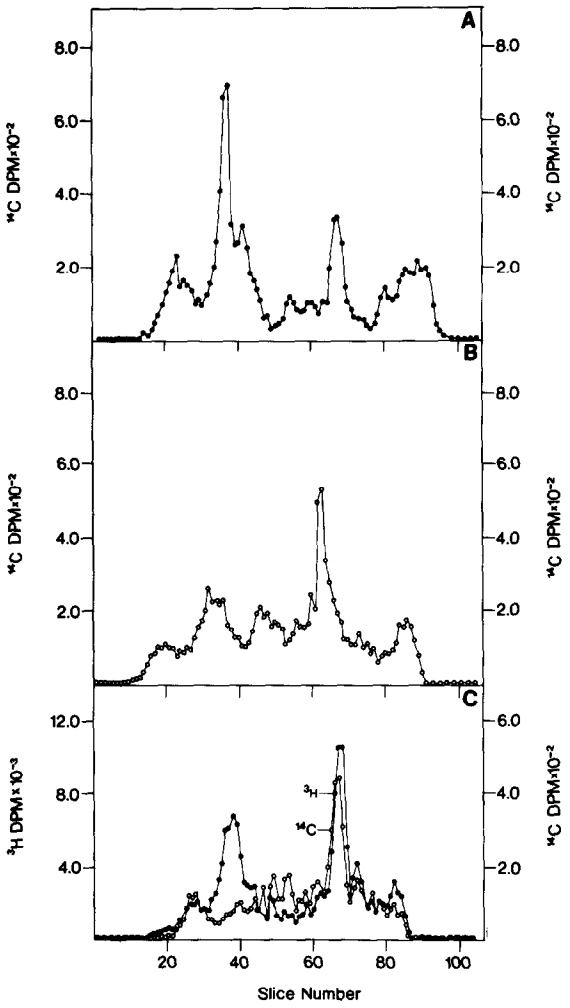

Fig.3. SDS-urea polyacrylamide gel electrophoresis of myosins in which all the thiol groups were blocked using iodo-[^{3}H] acetate or iodo-[^{14}C] acetate. About 800 μg protein containing about 250 000 dpm ^{3}H or 25 000 ^{14}C were applied to 0.7 × 12 cm disk gels which were prepared and analyzed for radioactivity as described in fig.2. (A) Myosin-N. (B) Myosin-T. (C) Co-electrophoresis of [^{3}H]carboxymethylated myosin-N (•) and [^{14}C]carboxymethylated myosin-T (○).

to electrophoresis (fig.2(C)). The difference in distribution of labeled cysteine peptides from myosin-N and myosin-T was again evident. Since the cysteine content of these myosins is the same [6,8], these results suggest that there are amino acid substitutions in myosin-T methionine residues which alter the mass of a cysteine containing peptide(s).

The experiments reported here indicate that thyroid hormone administration induces the synthesis of a new myosin species within the heart. This provides a satisfactory explanation for the change in enzymatic properties of cardiac myosin that have been observed in thyrotoxic animals.

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