Relative synthesis of cardiac contractile proteins

Evidence for synthesis from the same precursor pool

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The relative molar synthesis of cardiac contractile proteins has been measured in the perfused heart under control haemodynamic conditions. This synthesis, of myosin heavy chains, individual light chains (1 and 2), actin and tropomyosin, was determined from isolated guinea-pig hearts perfused for 3 h simultaneously with constant specific radioactivities and concentrations of [³H]lysine and [³H]phenylalanine. The data strongly suggest that all of the proteins studied were synthesized from the same precursor pools of lysine and phenylalanine, since the ratio of the specific activities of the two labels was the same in all of the proteins. Measurement of molar synthesis of each contractile protein was the same with either labelled amino acid. Under control haemodynamic-perfusion conditions, the relative molar synthesis of the contractile proteins was actin > heavy chains > light chain 2 > light chain 1 > tropomyosin.

Under various forms of cardiac stress, increases in total protein synthesis have been observed (Gudbjarnason *et al.*, 1964; Schreiber *et al.*, 1966, 1971, 1975; Hjalmarson & Isaksson, 1972; Sender & Garlick, 1973; Sanford *et al.*, 1978; Everett *et al.*, 1979), but there is little information concerning the synthesis of the individual contractile proteins. This is of particular importance, since reports have indicated that a different heavy-chain isoenzyme of myosin is synthesized under prolonged stress (Morkin, 1979; Hoh & Egerton, 1979; Lompre *et al.*, 1979). The relative synthesis of different proteins may be estimated from assays of turnover in a steady state *in vivo* (Arias *et al.*, 1969), and this has been applied to cardiac contractile proteins (Zak *et al.*, 1977). However, in response to stress the heart may not be in a steady state with respect to protein metabolism, and turnover may not give an accurate estimate of synthesis. Synthesis of cardiac protein may be measured from incorporation of labelled amino acids into protein by using continuous perfusion with constant specific radioactivity of labelled amino acids and is applicable to both steady and non-steady states.

Although the assay of synthesis requires data concerning the specific radioactivities of the labelled

amino acids in the precursor pool, these data are not required if relative synthesis of the proteins is measured and if the precursor pool for the synthesis is the same for all the proteins studied. The present paper describes such measurement of cardiac contractile-protein synthesis in the isolated perfused heart under control haemodynamic conditions.

Methods

Perfusion methods

Hearts from male guinea pigs (300–700 g) were used for perfusion. The operative procedures and perfusate were described previously (Schreiber *et al.*, 1973, 1977), with modifications that the right heart was converted into a closed chamber receiving only the coronary vein flow and the proximal pulmonary artery was cannulated to collect all of this flow. A perfusion load of 7–8 ml/min per g was presented to the left ventricle. The stroke volume of 0.03–0.04 ml was ejected by the ventricle against a controlled aortic pressure of 35–40 mmHg (4.67–5.33 kPa), similar to that found in anesthetized animals of the same size *in vivo* (Schreiber *et al.*, 1975). Ventricular ejection pressure, amplitude of ventricular contraction and $dP/dt(\max.)$ were monitored with the aid of a pressure transducer (Statham Instrument Co., type P 23 DB) directly in line with the aortic outflow. With this model, cardiac protein

Abbreviation used: SDS, sodium dodecyl sulphate.

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synthesis is linear for 3 h (Schreiber *et al.*, 1966, 1971).

The hearts were perfused for 3 h with both L-[4,5- ^3H]lysine and L-phenyl[2,3- ^3H]alanine at constant specific radioactivities within each experiment (7.5–25 mCi of [^3H]lysine and 3.6–10.9 mCi of phenyl[^3H]alanine/mmol) and constant concentration (0.2 mM-lysine and 0.46 mM-phenylalanine). The [^3H]lysine and phenyl[^3H]alanine were tested for purity before use by amino acid analysis. Only batches with more than 90% of the total radioactivity in the appropriate peak were used in the study. No evidence of D-lysine was found with L-lysine decarboxylase (Hutzler *et al.*, 1967).

Isolation of myosin, actin and tropomyosin

At the end of the perfusion, the left ventricle and septum were separated from the remainder of the heart, weighed and treated as reported for isolation of myosin by fractionation with saturated $(\text{NH}_4)_2\text{SO}_4$ (Evans *et al.*, 1978). The fractions collected were 25–35%, 35–42%, and 42–65% with respect to $(\text{NH}_4)_2\text{SO}_4$ saturation. The proteins obtained from each fraction were applied to SDS/polyacrylamide gels (Evans *et al.*, 1978) and the protein bands separated by molecular weights (Weber & Osborn, 1975), or were applied to similar gels containing 5 M-urea (Sender, 1971; Cummins & Perry, 1974). The bands for myosin heavy chains derived from all $(\text{NH}_4)_2\text{SO}_4$ fractions were divided into a trailing (cathode side) and a leading (anode side) portion in order to compare their specific radioactivities. Light chain 1 (mol.wt. 28 000) and light chain 2 (mol.wt. 20 000) (Evans *et al.*, 1978) were isolated from gels obtained from all $(\text{NH}_4)_2\text{SO}_4$ fractions; actin (mol.wt. $44\,700 \pm 400$) was obtained from the 25–35%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction (Szent-Gyorgyi, 1947), and tropomyosin from the 42–65%-satd. $(\text{NH}_4)_2\text{SO}_4$ fractions (Reddy & Honig, 1972; Fenner *et al.*, 1975). The identity of tropomyosin was established by gel electrophoresis in the presence (apparent mol.wt. of tropomyosin $55\,000 \pm 900$) and absence of 5 M-urea (apparent mol.wt. of tropomyosin $38\,000 \pm 600$), since the mobility of tropomyosin is retarded in urea without the mobilities of the other proteins being affected (Sender, 1971; Cummins & Perry, 1974). The bands of each protein were pooled, treated and hydrolysed as described by Weber & Osborn (1975), and the supernatant fluids passed through a column containing 2 g of Bio-Rad AG 50W (X4) resin to remove the Coomassie Blue dye. The amino acids were eluted with 1 M-NaOH, washed with water until the pH was neutral, and the pooled effluents flash-evaporated. The dried hydrolysates were dissolved and were analysed on an amino acid analyser (model no. 119; Beckman), and radioactive counting and measurement of the efficiency of the system were as

previously described (Schreiber *et al.*, 1973, 1975; Evans *et al.*, 1978).

Results

Purity of proteins was established from amino acid molar ratios and constancy of specific radioactivities of the labelled amino acids in the proteins isolated from the different $(\text{NH}_4)_2\text{SO}_4$ fractions.

Purity of proteins

(1) *Amino acid molar ratios.* A criterion of purity of the isolated proteins was the constancy of the amino acid ratios in each protein (expressed as the ratio amino acid/lysine; Table 1). The number of residues of lysine/molecule of protein are given in the legend to Table 1 for those proteins that have been isolated and hydrolysed in the absence of gel. The validity of the determinations is supported by the assays of bovine albumin, which were the same as those reported by Brown (1977). The data for heavy chains compares favourably with those previously described (Evans *et al.*, 1978). Molar ratios in actin found in the present study were similar to those obtained in guinea-pig cardiac actin isolated by the method of Spudich & Watt (1971) or from other species (Katz & Carsten, 1963; Carsten & Katz, 1964; Raszowski *et al.*, 1977). Light chain 1 and light chain 2 amino acid molar ratios assayed from gels as described above were the same as those isolated by elution with NH_4HCO_3 by the methods of Weber & Osborn (1975) (Evans *et al.*, 1978). Finally, the amino acid molar ratios in tropomyosin isolated by gel electrophoresis in the presence of urea were similar to those reported for cardiac tropomyosin (Cummins & Perry, 1974; Fenner *et al.*, 1975).

(2) *Specific radioactivity of the contractile proteins after 3 h of perfusion.* Comparison of the specific radioactivities of [^3H]lysine and phenyl[^3H]alanine in the heavy chains from the different $(\text{NH}_4)_2\text{SO}_4$ fractions agreed with each other ($\pm 6\%$) and there was no difference in the cathode or anode side of the heavy chains gel bands, indicating purity of the heavy chains. Free heavy chains have been reported to precipitate in the 25–35%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction (Wikman-Coffelt *et al.*, 1973a), but the present data do not support the existence of a free-heavy-chains pool unless this pool is rapidly exchangeable with the heavy chains in the assembled myosin.

Contamination of the light chains with similar-molecular-weight troponin components (Dabrowska *et al.*, 1973; Tsukui & Ebashi, 1973) was minimized by precipitating intact myosin in 35–42%-satd. $(\text{NH}_4)_2\text{SO}_4$ (Wikman-Coffelt *et al.*, 1973b), since troponin is precipitated at higher saturations (Reddy & Honig, 1972). The specific

Table 1. *Amino acid molar ratios with respect to lysine for the contractile proteins*

The number of lysine residues/molecule of protein were: for heavy chains, 150; actin, 19; light chain 1, 33; and light chain 2, 17. Values in parentheses are S.E.M.; those without S.E.M. are means of two values. Arginine values were frequently lost due to the high NH_3 content of the samples hydrolysed from gels. Owing to the large amount of sample applied to the column for actin to ensure an accurate phenylalanine value, aspartic acid was not always sufficiently resolved from the unknown peak, due to hydrolysis of gels, that eluted with the column bed volume.

Amino acid	Protein ... Average no. of determinations	Heavy chains ... 28	Tropomyosin 3	Actin 7	Light chain 1 9	Light chain 2 9
Asp		1.15 (± 0.01)	0.972 (± 0.025)	2.43	1.33 (± 0.07)	1.84 (± 0.08)
Thr		0.409 (± 0.017)	0.325	1.34 (± 0.07)	0.617 (± 0.041)	0.685 (± 0.031)
Ser		0.543 (± 0.015)	0.822	1.48 (± 0.07)	0.634 (± 0.073)	0.839 (± 0.106)
Glu		2.02 (± 0.07)	2.05 (± 0.27)	2.14 (± 0.08)	1.57 (± 0.04)	1.70 (± 0.08)
Pro		0.180 (± 0.006)	0.072 (± 0.045)	1.03 (± 0.12)	0.714 (± 0.033)	0.453 (± 0.023)
Gly		0.479 (± 0.027)	0.681 (± 0.187)	1.87 (± 0.12)	1.41 (± 0.08)	1.91 (± 0.018)
Ala		0.957 (± 0.030)	1.22 (± 0.072)	1.72 (± 0.04)	1.19 (± 0.06)	1.42 (± 0.11)
Val		0.363 (± 0.008)	0.293 (± 0.006)	0.854 (± 0.036)	0.494 (± 0.023)	0.432 (± 0.031)
Ile		0.390 (± 0.010)	0.293 (± 0.003)	1.05 (± 0.05)	0.359 (± 0.014)	0.433 (± 0.008)
Leu		1.13 (± 0.02)	0.848 (± 0.026)	1.37 (± 0.03)	0.804 (± 0.014)	0.706 (± 0.021)
Phe		0.299 (± 0.005)	0.085 (± 0.013)	0.590 (± 0.014)	0.485 (± 0.013)	0.743 (± 0.008)
Arg		0.582 (± 0.010)	—	0.913	0.417	0.232

Table 2. *Specific radioactivities of phenylalanine and lysine in cardiac contractile proteins*

Results are means for six studies in each case with simultaneous perfusion with [^3H]lysine and phenyl[^3H]alanine. Numbers in parentheses indicate the number of assays. Values are expressed as means $\pm$ S.E.M. and were calculated individually from the data for each experiment. The specific radioactivities of lysine and phenylalanine were constant in any one experiment, but different on different days. In order to compare the specific radioactivities of the proteins in all experiments, the data were normalized to a perfusate specific radioactivity of 5×10^6 c.p.m./ μmol for each labelled amino acid. Thus the Lys/Phe specific-radioactivity ratio was 1.0 for the perfusate. In six experiments the Lys/Phe specific-radioactivity ratio in the mixed intracellular pool was 0.863 ± 0.036 .

Protein ...	$10^{-4} \times$ Specific radioactivity (c.p.m./ μmol of amino acid)				
	Heavy chains	Light chain 1	Light chain 2	Actin	Tropomyosin
Lysine	1.81 ± 0.10 (6)	1.12 ± 0.07 (6)	1.38 ± 0.18 (6)	1.47 ± 0.12 (6)	2.38 ± 0.14 (5)
Phenylalanine	1.83 ± 0.14 (6)	1.19 ± 0.12 (6)	1.50 ± 0.14 (6)	1.56 ± 0.14 (6)	1.99 (2.05, 1.93)
Mean all	1.82 ± 0.08 (12)	1.16 ± 0.07 (12)	1.44 ± 0.11 (12)	1.52 ± 0.09 (12)	2.27 ± 0.12 (7)
Lys/Phe	1.00 ± 0.03	0.976 ± 0.083	0.919 ± 0.068	0.946 ± 0.020	1.11
S_1/S_2^*	—	0.638 ± 0.033	0.786 ± 0.038	0.845 ± 0.052	1.35 ± 0.05

* S_2 , specific radioactivity of the heavy chains; S_1 , specific radioactivity of light chain 1, light chain 2, actin or tropomyosin.

radioactivities of light chain 1 and light chain 2 obtained from gel electrophoresis and precipitation in 25–35% and 35–42% satd. $(\text{NH}_4)_2\text{SO}_4$ fractions

were the same ($\pm 6\%$) and the data were pooled. Specific-radioactivity values for the two labelled amino acids are listed in Table 2.

Relative molar synthesis of the contractile proteins

When incorporation of labelled amino acids is used to measure protein synthesis, the specific radioactivity of the newly synthesized protein is dependent on the dilution with pre-existing unlabelled protein, and the relative molar synthesis of any two proteins must also consider this dilution. For example, if we are measuring the amount (in μmol) of heavy chains synthesized with lysine as a label, then:

$$S = a^*/(A + a)$$

where S is the specific radioactivity of lysine (c.p.m./ μmol) in the heavy chains, a^* is the total amount of incorporated radioactivity, A is the total amount of lysine in the heavy chains at zero time, and a is the amount of lysine incorporated into newly synthesized heavy chains at time t . Since the amount of protein synthesized in 3 h is very small compared with the total amount of protein present (a is less than 0.2% of A), then:

$$S = a^*/A \quad (1)$$

If the same precursor pool is used for the synthesis of two proteins, their relative molar synthesis (M_{s1}/M_{s2}) may be determined from the specific radioactivities of the two proteins (S_1 and S_2) and the molar proportions of the proteins in the tissue (M_1 and M_2), and:

$$\frac{M_{s1}}{M_{s2}} = \frac{S_1}{S_2} \times \frac{M_1}{M_2} \quad (2)$$

Evidence that the precursor pool for the different contractile proteins has the same specific radioactivity was found with simultaneous perfusion of the hearts with the two labelled amino acids. Comparison of the specific-radioactivity ratios of [^3H]lysine/phenyl[^3H]alanine showed that these ratios were essentially the same in all of the proteins assayed in the left ventricle (Table 2). This would indicate synthesis from pools having the same ratio of specific radioactivities or the same precursor pool.

In view of the above, it was then valid to assay S_1/S_2 for each labelled amino acid in the contractile proteins, where S_1 represented the specific radioactivity of the label in light chain 1, light chain 2, actin or tropomyosin, and S_2 represented the specific radioactivity of the label in heavy chains (Table 2). However, S_1/S_2 does not give any indication of the quantities synthesized, since the molar proportions of the contractile proteins are not the same (Katz, 1970, 1977; Fredericksen *et al.*, 1978), and dilution of the newly synthesized proteins by different proportions of pre-existing proteins had to be taken into account. Hence, molar synthesis was calculated (eqn. 2) by using S_1/S_2 values determined in the

present study multiplied by the values of molar proportions (M_1/M_2) for cardiac muscle. The molar proportions of the contractile proteins were calculated from available data for cardiac muscle and molecular weights (myosin 500 000, actin 42 000, and tropomyosin 70 000). These were: heavy chains 3.04; light chain 1 3.04; light chain 2 3.04; actin 7.0; tropomyosin, 1.2 (Katz, 1970, 1977; Fredericksen *et al.*, 1978; Weeds & Frank, 1973).

By using the above proportions, the relative molar synthesis was actin > heavy chains > light chain 2 > light chain 1 > tropomyosin (1.94 actin:1 heavy chains:0.79 light chain 2:0.64 light chain 1:0.53 tropomyosin).

Discussion

Measurement of relative synthesis of specific proteins avoids the problem of assaying the precursor-pool specific radioactivity, provided that the precursor pool for the synthesized proteins is the same. In some tissues there may be more than one precursor pool for protein synthesis (Ilán & Singer, 1975; Fern & Garlick, 1976). However, this does not appear to be the case in the present study, since the identical ratio of specific radioactivities (lysine/phenylalanine) in each protein measured indicated that the synthesis proceeded from a single pool or from pools that have identical physiological and kinetic behaviour.

Of further interest in the present study, the ratio of the specific radioactivities of the two amino acids in the contractile proteins suggests greater contribution to the precursor pools of both amino acids from the extracellular space than from the mixed intracellular (acid-soluble) pool. Similar unequal contributions have been indicated (Hider *et al.*, 1971; Ilán & Singer, 1975; Vidrich *et al.*, 1977; McKee *et al.*, 1978).

The ratio of specific radioactivities of two proteins (S_1/S_2) may be equated with their relative fractional rates of synthesis (k_{s1}/k_{s2}). However, in view of the different molar proportions of the contractile proteins within the cell, the use of relative fractional rates of synthesis does not indicate the quantities of protein synthesized. The latter may be estimated with relative molar synthesis (M_{s1}/M_{s2}).

The pattern of molar synthesis found in the present study, of actin > heavy chains > light chain 2 > light chain 1 > tropomyosin, is quite different from that observed with fractional rates of synthesis in the present study (S_1/S_2) and that reported for turnover rates *in vivo* (Zak *et al.*, 1977). However, if one uses the molar proportions of the cardiac proteins described above and the fractional rates of degradation (derived from the half-lives reported by Zak *et al.*, 1977) to calculate molar degradation, then the calculated molar degradation proceeds in

the order actin > heavy chains > light chain 2 = light chain 1 > tropomyosin. Since degradation should equal synthesis in the steady state, the calculations are in essential agreement with the findings of the present study.

It was noteworthy that two proteins light chain 1 and light chain 2 having the same molar proportions as heavy chains in the myosin molecule (Weeds & Frank, 1973) had S_1/S_2 values less than unity. This would imply either the presence of an intracellular light-chain pool (Zak *et al.*, 1977; LaGrange & Low, 1976) or actual differences in the rates of synthesis of the two proteins. The latter does not compromise the maintenance of myofibrillar composition, since, in a steady state, a protein with relatively increased synthesis would have increased degradation. In this respect, it should be noted that the relative higher synthesis of heavy chains compared with light chains reported in the present study and previously in the right ventricle (Evans *et al.*, 1978) is paralleled by similar findings for degradation of these proteins *in vivo* (Zak *et al.*, 1977). It was also noteworthy that the molar-synthesis data indicate that, for every seven actin molecules, 1.9 tropomyosin molecules are made, a value higher than indicated by the molar proportions in the myofibril. It may be suggested that greater availability of tropomyosin aids in assembly of the thin filament, since it has been noted to improve conversion of globular actin to fibrous actin *in vitro* (Weber & Murray, 1973). However, in the absence of data on assembly, the existence of the latter phenomenon is speculative.

In summary, the present study provides results concerning molar synthesis of contractile proteins measured *in vitro* by the continuous-perfusion method. The relative molar synthesis of the contractile proteins is actin > heavy chains > light chain 2 > light chain 1 > tropomyosin, and the syntheses appear to be carried out from the same or identically labelled precursor pools.

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