Turnover of the creatine kinase subunits in chicken myogenic cell cultures and in fibroblasts

Mario CARAVATTI* and Jean-Claude PERRIARD†

Institut für Zellbiologie, Eidgenössische Technische Hochschule Zürich, Hönggerberg, CH-8093 Zürich, Switzerland

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The rates of degradation of creatine kinase subunits, M-CK and B-CK subunits, were measured in cultured myogenic cells and in subcultured fibroblasts. In differentiated myogenic cells, the myotubes, both M-CK and B-CK subunits are synthesized. Their rates of degradation were compared. The M-CK subunit is slightly more stable and is degraded with an average apparent half-life of 75 h, whereas that of the B-CK subunit was shorter with 63 h. The turnover properties of M-CK subunit from soluble and of myofibril-bound MM-CK homodimeric creatine kinase isoenzyme isolated from breast muscle of young chickens were identical. The apparent half-life of the B-CK subunit was also determined in subcultured fibroblasts and 5-bromo-2'-deoxyuridine-treated cells, and found to be shorter than in myotubes (46 h and 37 h respectively). Similar observations were made for myosin heavy chain, actin and total acid-precipitable material. It appears therefore that proteins are in general degraded more slowly in differentiated myogenic cells. The differences in the stability of M-CK and B-CK subunits in myotubes probably do not reflect a major regulatory mechanism of the creatine kinase isoenzyme transition.

The transition of creatine kinase from the BB-CK isoenzyme to the MM-CK isoenzyme is known to occur in developing embryonic muscle (Eppenberger *et al.*, 1964; Perriard *et al.*, 1978) as well as in cultured myogenic cells (Turner *et al.*, 1974; Perriard *et al.*, 1978). This system was chosen to study the events involved in the regulation of muscle-specific gene expression. Many studies so far have discussed the possibility that accumulation of specific proteins during terminal differentiation of myogenic cells is not solely a reflection of transcriptional activity, but can also be effected at a variety of steps during the biogenesis of these molecules, for example at post-transcriptional (Buckingham *et al.*, 1976; Bag & Sarkar, 1976; Dym *et al.*, 1979) or at translational (Heywood *et al.*, 1974; Kennedy *et al.*, 1978) control levels. In addition, the importance of post-translational control as a regulatory process controlling tissue-

specific isoenzyme pattern has been examined by several authors. Fritz *et al.* (1971, 1973) proposed that preferential degradation of different subunit types of lactate dehydrogenase is of major importance in regulating isoenzyme patterns in mammalian cells. The same mechanism is suggested to bring about the observed changes in lactate dehydrogenase isoenzyme patterns during differentiation of bone-marrow erythroid cells *in vitro* (Setchenska & Arnstein, 1978). In the case of creatine kinase isoenzymes the decrease in the concentrations of the BB-CK isoenzyme, found in embryonic muscle and many adult tissues, and the hybrid MB-CK isoenzyme during development of rabbit skeletal muscle was assumed to be associated with a rapid turnover of the B-CK-subunit-bearing isoenzymes (Perry, 1971). The selective inactivation of the BB-CK isoenzyme was thought to be responsible for the disappearance of its activity (Armstrong *et al.*, 1977a) during development of rabbit muscle. Such observations lead to the possibility that isoproteins found in embryonic undifferentiated tissues are degraded much faster than are the isoproteins typical of the differentiated tissues, a mechanism that was also postulated for the putative 26S mRNA for myosin heavy chain (Buckingham *et al.*, 1976).

Abbreviations used: M-CK and B-CK subunits, subunits of creatine kinase; MM-CK and BB-CK isoenzymes, homodimeric creatine kinase; MB-CK isoenzyme, heterodimeric creatine kinase.

* Present address: Institut de Biologie Moléculaire, Institut Pasteur, Paris, France.

† To whom correspondence should be addressed.

In the present paper we examine whether post-translational control plays an important role in the regulation of the creatine kinase isoenzyme transition.

The myotubes arising by the differentiation of myogenic cells *in vitro* contain all three isoenzymes of creatine kinase (Turner *et al.*, 1974; Perriard *et al.*, 1978) and actively synthesize both types of subunits, M-CK and B-CK (Caravatti *et al.*, 1979). These cells are therefore useful for the study of the question of differential degradation of the creatine kinase isoenzymes. A comparison with the metabolism of creatine kinase in fibroblasts and 5-bromo-2'-deoxyuridine-suppressed myogenic cells is described.

Similar rates of degradation were measured in pulse-chase experiments for both types of creatine kinase subunits, B-CK and M-CK. The results suggest that differential degradation does not govern the transition from BB-CK isoenzyme to MM-CK isoenzyme in myogenic cells, but may slightly modulate the concentrations of creatine kinase in myogenic cells differentiating *in vitro*.

Materials and methods

Materials

Fertilized White Leghorn eggs were obtained from Geflügel Wolff (Volketswil, Switzerland). L-[³H]Leucine (35–50 Ci/mmol), L-[¹⁴C]leucine (340 mCi/mmol) and [¹⁴C]thymidine (460 mCi/mmol) were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.), and 5-bromo-2'-deoxy[¹⁴C]uridine (40–60 mCi/mmol) was from NEN (Dreieich, Germany). Acrylamide and bisacrylamide were from Serva, minimal essential medium with Earle's salts, horse serum, antibiotic/antimycotic mixture and glutamine solution were obtained from Flow Laboratories (Irvine, Scotland, U.K.). Trypsin (Bacto) was from Difco Laboratories (Detroit, MI, U.S.A.) and 5-bromo-2'-deoxyuridine was from Calbiochem (Luzern, Switzerland).

Cell cultures

Primary cultures of myogenic cells were prepared from 11–12-day chicken embryos as described by Turner *et al.* (1974). Cells were plated in gelatinized 100 mm Falcon tissue-culture dishes at a density of 2×10^5 cells/ml in 8 ml of standard medium containing 10 parts of horse serum, 3.5 parts of embryo extract and 86.5 parts of minimal essential medium with Earle's salts, supplemented with glutamine and antibiotics. In some experiments differentiation of myogenic cells was suppressed by 5-bromo-2'-deoxyuridine (2.5 µg/ml), a thymidine analogue, added at the time of plating (O'Neil & Stockdale, 1974). Secondary fibroblast cultures were obtained by subculturing myogenic cultures overgrown with

fibroblasts by mild trypsin treatment as described by Turner *et al.* (1974). During chase periods fibroblasts as well as 5-bromo-2'-deoxyuridine-suppressed cells were confluent and comparable with the non-dividing myotubes.

Labelling of cells

For the measurement of rates of degradation of the creatine kinase subunits, cultures were labelled for 12 h in 4 ml of labelling medium containing [³H]leucine at 15 µCi/ml and only 20% of the usual leucine content. At the end of the labelling period, cells were rinsed once with 8 ml of sterile Simm's balanced salt solution (Simm & Sanders, 1942) containing 10 mM-leucine, and then received 8 ml of standard medium containing an additional 10 mg of leucine/ml in order to minimize isotope re-utilization. Cells were allowed to equilibrate in the new medium for 6 h (= zero time), and then harvested at different time intervals and stored frozen at –20°C.

For these experiments it was important to determine whether any cells had been lost from the culture dish during the course of the experiments. Therefore all cultures were prelabelled with [¹⁴C]thymidine (0.4 µCi/ml) (5-bromo-2'-deoxyuridine-treated cultures with 5-bromo-2'-deoxy[¹⁴C]uridine) from the time of plating up to the beginning of the protein-labelling period with [³H]leucine. The ¹⁴C content in the cultures at any time during the chase period was used as an estimate for cell recovery (Rubinstein *et al.*, 1976) and served to correct the results for cell loss.

Administration of radioactive leucine to chickens

After overnight deprivation of food, 3-day-old chickens received 500 µCi of [³H]leucine in 150 mM-NaCl/50 mM-sodium phosphate buffer, pH 7.2, by intraperitoneal injection. After 6 days, 60 µCi of [¹⁴C]leucine was injected and the animals were killed 24 h later (Lebherz, 1975). The musculus pectoralis major muscles were immediately cut out, cleaned free of connective tissue and immersed in 20 vol. of the contraction-inhibiting solution A (0.1 M-KCl/1 mM-EGTA/1 mM-dithiothreitol/5 mM-EDTA, pH 7.0) (Wallimann *et al.*, 1977) before the preparation of myofibrils or total extract.

Extraction and purification of proteins

For the isolation of creatine kinase subunits, myosin heavy chain and actin, cells were scraped from the culture dishes in 2 mM-potassium phosphate buffer, pH 6.8, containing 1 mM-2-mercaptoethanol and 20 mM-KCl, and sonicated for four 10 s periods (MSE 100 W ultrasonic disintegrator at maximal output fitted with a small probe). A 100 µl portion of the homogenate was extracted with the same buffer for 1 h at 4°C, and cell debris was removed by centrifugation at 25 000 g for 30 min at

4°C. The radioactivity incorporated into creatine kinase subunits isolated by specific immunoprecipitation was determined as described by Caravatti *et al.* (1979).

Actomyosin was prepared by using the procedure of Adelstein *et al.* (1972). Portions (200 µl) of cell homogenates were adjusted to 0.6M-KCl, 10mM-dithiothreitol and 15mM-potassium phosphate buffer, pH 7.5, extracted with this buffer for 4h and cleared by centrifugation at 25 000g for 30min at 4°C. After addition of partially purified carrier actomyosin from unlabelled breast muscle to the supernatant, actomyosin was precipitated by dilution with 6vol. of 2mM-MgSO₄/10mM-potassium phosphate buffer, pH 6.0, and collected by centrifugation at 25 000g for 20min. The precipitated actomyosin was further fractionated on polyacrylamide slab gels (8% acrylamide) in the presence of 0.1% sodium dodecyl sulphate (Laemmli, 1970). The actin and myosin bands were cut from the gels, and radioactivity was determined in a Packard Tri-Carb 2650 scintillation spectrometer as described by Caravatti *et al.* (1979).

Incorporation of [³H]leucine or [¹⁴C]thymidine into total acid-precipitable material was determined by precipitating samples of homogenates with 10% (w/v) trichloroacetic acid on paper discs, which were washed batchwise successively with 5% trichloroacetic acid, 95% (v/v) ethanol and ether (Roberts & Paterson, 1973). The ³H and ¹⁴C radioactivities were counted simultaneously and radioactivity measurements were corrected for counting efficiency by the external-standards-ratio method.

Preparation and extraction of myofibrils

Myofibrils were prepared (all steps at 4°C) from chicken breast muscle, musculus pectoralis major, as described by Wallimann *et al.* (1977). Exhaustively washed myofibrils were extracted in low-salt buffer (5mM-Tris/HCl buffer, pH 7.7, containing 1mM-dithiothreitol) and centrifuged at 5000g for 10min to separate insoluble matter from released proteins containing the myofibrillar MM-CK isoenzyme.

Results

The rate of loss of radioactivity from creatine kinase subunits was measured in a series of experiments in differentiated myogenic cultures, 5-bromo-2'-deoxyuridine-suppressed cultures and subcultured fibroblasts.

Cell loss, especially from cultures of differentiated contracting myotubes, from the culture dishes during the experiments represented a technical problem. By prelabelling the dividing cells with [¹⁴C]-nucleotides it was possible to get an estimate of the number of labelled nuclei present at any time

from the ¹⁴C radioactivity remaining in the culture dishes during the chase period (results not shown). There appears to be more cell loss in differentiated myogenic cultures, possibly due to spontaneous contractions eventually leading to detachment from the dishes, than in 5-bromo-2'-deoxyuridine-suppressed myogenic cultures and in fibroblast subcultures. These values were used to discriminate between the decrease of radioactivity from protein due to protein degradation and that due to cell loss.

The degradation of creatine kinase subunits, myosin heavy chain, actin and total acid-precipitable protein followed in all cases pseudo-first-order kinetics. As shown in Fig. 1, the individual values fit the calculated linear regression lines in the semi-logarithmic plot very well. The half-lives of these components could be derived from the decay of radioactivity (Table 1) during the chase period. Thus in these experiments M-CK and B-CK subunits are degraded with half-lives of about 75h and 63h respectively in differentiated myogenic cultures. Half-lives comparable with that of B-CK subunit were found for myosin heavy chain and actin in these cultures.

In 5-bromo-2'-deoxyuridine-suppressed myogenic cultures and fibroblast subcultures B-CK subunit as well as myosin heavy chain and actin are degraded with half-lives considerably shorter than in differentiated myogenic cultures. However, it should be noted that it is difficult to decide whether only these components are effectively degraded with enhanced rates in these cultures, since total acid-precipitable material was degraded with shorter half-lives. Therefore it is rather likely that the protein degradation in fibroblasts and 5-bromo-2'-deoxyuridine-treated cells is faster than in differentiated myogenic cells.

The results indicate that B-CK subunits have a 15% shorter half-life than do M-CK subunits in differentiated myogenic cultures. The value for the B-CK subunit, however, represents not only the B-CK subunits of the myotubes, but also the B-CK subunits synthesized by contaminating fibroblasts. These cells have been shown to synthesize B-CK subunit at a 10–15-fold lower maximal rate than do differentiated myogenic cells (Caravatti *et al.*, 1979), and also have been shown to degrade B-CK subunit with a much shorter half-life, about 46h (Table 1). A slightly lower value for half-life of B-CK subunits in differentiated myogenic cultures had therefore to be expected, even if the two subunits of myotubes had identical values for their rates of degradation. If there were a minor difference in the susceptibility to degradation of B-CK and M-CK subunits, the more acidic isoelectric point of B-CK subunit (Rosenberg & Perriard, 1981) would be consistent with the correlation between charge and stability shown previously (Dice & Goldberg, 1975).

Fig. 1. *Degradation of creatine kinase subunits B-CK and M-CK, actin, myosin heavy chain and total protein* Cultures prelabelled with [^{14}C]thymidine (see the text) were labelled at 68 h in culture with 15 Ci of [^3H]leucine/ml for 12 h and changed to non-radioactive medium afterwards as described in the Materials and methods section. At the time points after plating indicated, the radioactivity remaining in B-CK subunit ($\bullet$), M-CK subunit ($\blacktriangle$), actin ($\diamond$), myosin heavy chain (∇) and total protein ($\blacksquare$) was measured. Each value represents the average of two determinations and is corrected for cell loss. The lines are calculated regression lines for the values shown. (a) Standard myogenic culture; (b) 5-bromo-2'-deoxyuridine-suppressed myogenic culture; (c) fibroblast subculture.

Table 1. *Half-lives of B-CK isoenzyme, M-CK isoenzyme, actin, myosin heavy chain and total protein*

Cultures identical with those described in the Materials and methods section were labelled at 68 h (Expt. 1) and 58 h (Expt. 2) respectively with 15 μCi of [^3H]leucine/ml for 12 h and then switched to non-radioactive chase medium. Half-lives were calculated from the linear-regression analysis of the experimental values from decay curves (Fig. 1). Abbreviation: BrdUrd, 5-bromo-2'-deoxyuridine.

Cell cultures	Half-life (h)					
	Expt. no.	B-CK isoenzyme	M-CK isoenzyme	Actin	Myosin heavy chain	Total protein
Differentiated myogenic culture	1	62	72	50	50	55
	2a	65	74	65	71	51
	2b	62	79	55	56	45
BrdUrd-suppressed myogenic culture	1	36	—*	41	39	38
	2	38	—*	45	41	39
Fibroblast subculture	1	43	—*	40	32	45
	2	49	—*	43	28	48

* Cells do not contain any M-CK isoenzyme subunits.

In summary, it can be stated that B-CK and M-CK subunits are degraded with similar rates and that these rates could possibly turn out to be nearly identical within myotubes.

All these experiments were performed on the assumption that the creatine kinase subunits are evenly distributed throughout the cytoplasm and that all molecules are subject to random turnover. With the MM-CK isoenzyme, however, it has been shown that not all of the enzymic activity occurs as readily soluble activity, but rather that some of the MM-CK isoenzyme is bound in the M-line of myofibrils (Turner *et al.*, 1973; Wallimann *et al.*, 1977). We explored the question whether the sequestration of some of the MM-CK isoenzyme into the M-line-bound fraction leads to a change of its rate of degradation. Such an experiment is not easy to conduct in tissue-culture cells, since the myofibrils even of well-differentiated myotubes could not be purified (results not shown), and we had to perform this experiment with tissue from young chickens. These muscles allow easy purification of myofibrils. The experiment was performed by using the double-labelling technique of Arias *et al.* (1969).

Chicks received first an intraperitoneal injection of [^3H]leucine, followed 6 days later by an injection of [^{14}C]leucine. The animals were killed 24h after the last injection (Lebherz, 1975). The M-CK subunits were purified from the soluble MM-CK-isoenzyme pool and from the MM-CK isoenzyme released from thoroughly washed myofibrils. The $^{14}\text{C}/^3\text{H}$ ratios of radioactivities taken up into the proteins were determined. The ratio of the isotopes is an indication of the turnover characteristics of the components studied. High $^{14}\text{C}/^3\text{H}$ ratios indicate a high turnover, whereas low ratios indicate a rather stable fraction of proteins. By this technique no significant change in turnover characteristics due to sequestration of some of the MM-CK isoenzyme dimers could be observed in two separate and independent experiments shown in Table 2. The isotope ratios in both cases are almost equal for the M-CK subunit of the soluble fraction and the M-CK

subunit bound to myofibrils. This indicates that the M-CK subunit molecules of the two pools in question turn over with the same kinetics or that there is free exchange of the M-CK subunits or MM-CK isoenzyme dimers between these two pools.

There is no reason to believe that a much longer half-life of the bound myofibrillar MM-CK isoenzyme in the cell cultures would increase the average half-life of the total M-CK isoenzyme. More generally, our results agree with the observation that there is no difference in the rate of degradation of soluble and myofibrillar proteins in different muscle types (Millward, 1978).

Discussion

In the present paper it has been shown that creatine kinase subunits M-CK and B-CK are degraded at similar rates in differentiated myogenic cells. The minor differences in the half-lives of the creatine kinase subunits suggest that degradation does not play an important role as a regulatory process controlling the creatine kinase isoenzyme transition. The finding that B-CK subunits are degraded about 1.5 times as fast in 5-bromo-2'-deoxyuridine-suppressed myogenic cells and in subcultures of fibroblasts as in differentiated myogenic cells indicates that degradation is different for the same isoenzyme in different cell types, as is the degradation of total proteins, thus affecting concentrations of creatine kinase isoenzymes in various cell types.

The reasons for the discrepancy between our findings and those of other investigators that support post-translational control for the regulation of other creatine kinase isoenzymes are unclear (Perry, 1971; Armstrong *et al.*, 1977b). Our results were obtained with myogenic cell cultures, whereas others worked with whole muscle from rabbit, representing a combination of several cell types that may complicate the interpretation of the results (Lebherz, 1974). Armstrong *et al.* (1977b), who reported an inactivation of enzymic activity of BB-CK isoenzyme, used for the detection of inactive BB-CK isoenzyme an antibody preparation that did not cross-react with the MB-CK isoenzyme (Armstrong *et al.*, 1977a). This is clearly in contrast with other findings (Roberts *et al.*, 1976; Perriard *et al.*, 1978). Studies on other isoenzymes have generally given results that are in agreement with our results (Lebherz, 1975). Fritz *et al.* (1971) proposed that regulation of lactate dehydrogenase isoenzymes involves differential degradation, although Lebherz (1974) was able to explain their findings on the basis of tissue heterogeneity. Similarly, the regulation of the different fructose biphosphate aldolase tetramers in avian brain was found to be governed by the differential synthesis of the different types of

Table 2. Relative rates of turnover of total cellular and myofibril-bound MM-CK isoenzyme

Chicks received [^3H]leucine followed by [^{14}C]leucine 6 days later as indicated in the Materials and methods section.

Protein	Relative rate of turnover ($^{14}\text{C}/^3\text{H}$ ratio)	
	Expt. 1	Expt. 2
MM-CK isoenzyme: myofibril-bound	0.14	0.11
MM-CK isoenzyme: total cellular	0.12	0.11
Total soluble protein	0.27	0.26

subunits involved and not by differential degradation characteristics (Lebherz, 1975).

In conclusion, we assume that changes in the synthesis of creatine kinase subunits (Caravatti *et al.*, 1979) corresponding to alterations of the mRNA concentrations (Perriard, 1979) are the major parameters in determining changes in the accumulation pattern of creatine kinase isoenzymes during terminal differentiation of myogenic cells, rather than differential degradation.

During terminal differentiation of myogenic cells in culture, increases in the half-lives for actin and myosin heavy chain have been reported (Rubinstein *et al.*, 1976; Zani *et al.*, 1978). Heterogeneous degradation rates for actin and myosin heavy chains have been discussed in connection with differential degradation of different isoenzymic forms of muscle proteins and with the sequestration of these proteins into myofibrils resulting in enhanced stabilities (Rubinstein *et al.*, 1976; Zani *et al.*, 1978; Walker & Strohmman, 1978). The facts that the half-lives reported are quite different (for myosin heavy chain they lie between 48 and 200 h in differentiated myogenic cells), and that the methods used did not distinguish between non-muscle and muscle-specific forms of these proteins, make impossible in this case an exact statement about the importance of protein degradation in controlling muscle-specific protein accumulation.

From other studies done *in vivo* and *in vitro* there is evidence that rates of degradation of total protein can vary under different physiological and pathological conditions leading to alterations in protein accumulation (Rourke, 1975; Goldberg & St. John, 1976; Millward, 1978; Walker & Strohmman, 1978).

Although protein degradation may be an important component besides protein synthesis in the modulation of muscle protein mass, we conclude from our results that degradation of the non-muscle B-CK isoenzyme subunit does not play an important role in the regulation of the creatine kinase isoenzyme transition. Furthermore, no evidence could be found for a differential degradation of structurally bound M-CK isoenzyme subunit as compared with the soluble pool of enzyme.

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