BBA 66538

SENSITIVITY OF ADENYLATE KINASE ISOZYMES FROM NORMAL AND DYSTROPHIC HUMAN MUSCLE TO SULFHYDRYL REAGENTS

R. HEINER SCHIRMER AND EVA THUMA*

Max-Planck-Institut für Medizinische Forschung, 69 Heidelberg, Jahnstraße 29 (Germany)

(Received September 20th, 1971)

SUMMARY

1. Crystalline human muscle adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) possesses two sulfhydryl groups. The enzyme was only partially inactivated when these thiol groups were reacted with mercury or silver compounds. Reaction with Ellman's reagent, however, resulted in complete loss of activity.

2. The adenylate kinase activity of extracts of normal human muscle was inhibited by Ellman's reagent whereas the adenylate kinase activity of extracts of human liver was insensitive towards all sulfhydryl reagents.

3. In extracts of diseased human muscle (progressive muscular dystrophy Duchenne type) 40–60% of the adenylate kinase activity remained after treatment with Ellman's reagent.

4. These and other results indicate that the adenylate kinase isozymes might be an adequate system for investigating the role of SH-proteins in the pathogenesis of muscular dystrophies.

5. The reaction of mercury compounds with 2-nitro-5-thiobenzoate was used for the quantitative determination of organic mercurials.

INTRODUCTION

One of the remarkable facts in progressive muscular dystrophy is the occurrence of proteins which are not present in normal adult muscle[1,2]. KUBY and co-workers[3], for example, isolated from human dystrophic muscles a creatine kinase (ATP:creatine phosphotransferase, EC 2.7.3.2) which is normally only found in the brain. Obviously one would like to know if there might be a biological meaning in the production of isozymes under pathological conditions in the muscle. We tried to find an answer to this question in the following way: According to several authors[4,5] the basic bio-

Abbreviations: DTNB, 5,5'-dithiobis-(2-nitrobenzoate); PHMB, *p*-hydroxymercuribenzoate.

* The studies reported in this paper were taken from a thesis submitted by E. Thuma to the Medizinische Fakultät der Ruperto-Carola-Universität in Heidelberg.

chemical lesion in progressive muscular dystrophy could be the modification of SH groups which are essential for the function of certain proteins. Using this hypothesis we looked for an enzymic activity that can be inhibited by thiol reagents in normal muscle and that is carried out in dystrophic muscle by isoenzymes which are insensitive to the modification of SH groups. Adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) activity was found to fulfill these conditions.

RESULTS AND DISCUSSION

The adenylate kinase isozymes occurring in mammalian liver possess no free SH groups[6-8] whereas the set of isozymes present in healthy mammalian muscle is inactivated by certain sulfhydryl reagents[9,10]. To find the most suitable SH reagent for our studies, the predominant adenylate kinase isozyme in normal muscle[11], a purified crystalline protein, was reacted with $AgNO_3$, mercury compounds, and Ellman's reagent, respectively (Fig. 1). Only the latter proved to be satisfactory as a specific thiol reagent which completely inactivates the enzyme. This was confirmed by preparing human D-adenylate kinase[12,13], a stable, soluble and enzymatically inactive derivative in which both reactive sulfhydryl groups of the enzyme have been reacted with DTNB. Adenylate kinase activity could be completely regenerated by treating D-adenylate kinase with dithiothreitol (Fig. 1).

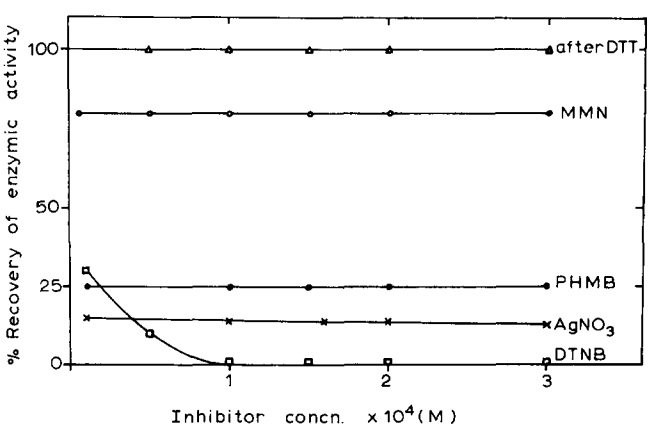


Fig. 1. Inhibition of human muscle adenylate kinase by sulfhydryl reagents. Adenylate kinase at $5 \cdot 10^{-6}$ M (= 200 enzyme units/ml = 100% enzymic activity) was incubated with various concentrations of sulfhydryl reagents in 1 ml of 0.2 M Tris–HNO_3 (pH 8.0) for 30 min at 4°. Then 5–20-μl samples were assayed for enzymic activity by the pH-stat procedure. Various degrees of inhibition were obtained with different sulfhydryl reagents. The possibility that the observed activity after treatment with PHMB or methylmercury nitrate (MMN) was due to the dissociation of the mercurial–protein complex in the assay mixture was excluded by using the microcolumn assay of NODA and co-workers[9] as an alternative procedure. Both methods gave identical results. If dithiothreitol (2 mM final concentration) was added to the assay mixture the enzyme activity was found to be fully restored ("after DTT", upper line).

Not only the enzymic activity of pure muscle adenylate kinase but also the adenylate kinase activity in an extract of normal human muscle was completely inhibited by ELLMAN's reagent (Fig. 2); the liver extract, however, was scarcely affected by 5,5'-dithiobis-(2-nitrobenzoate) (DTNB). (The loss of 15% of the activity

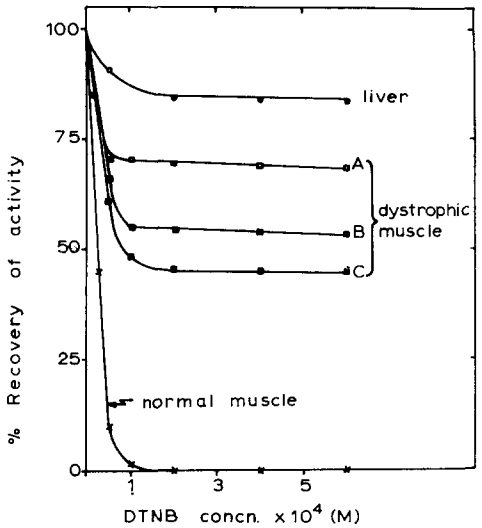


Fig. 2. Inhibition of the adenylate kinase activities of human liver, normal and dystrophic muscle by Ellman's reagent. The tissue extracts (10 units of adenylate kinase per ml of 0.1 M Tris–HNO_3, pH 8.0) were preincubated for 20 min at 25° with various concentrations of DTNB. For each sample a control without DTNB was prepared. Then 50 μl samples were tested for adenylate kinase activity by the pH-stat procedure. It should be noted that 100% enzyme activity is different for each tissue; the absolute values (expressed as enzyme units per g wet weight of tissue) were: 94 units/g of liver, 220 units/g of normal muscle, 120 units/g (A), 160 units/g (B) and 95 units/g (C) of dystrophic muscle. A, B and C represent tissues from three different patients. The normal muscle adenylate kinase isozyme could be completely inactivated by DTNB in all extracts: When 10 units/ml of pure human muscle adenylate kinase had been added to each extract before DTNB the plateaus of remaining activity were identical with those shown in the figure.

may be completely attributable to erythrocyte adenylate kinases which are likely to be present in the extract from liver and which are sensitive to DTNB (E. THUMA, unpublished results). In extracts of dystrophic muscles about 50% of the adenylate kinase activity remained after treatment with Ellman's reagent (Fig. 2).

In addition to the samples represented in Fig. 2, two specimens of dystrophic muscle were tested in which more than 80% of the muscle fibres had been replaced by fat and connective tissue cells. The extractable adenylate kinase activities were only 40 units/g and 20 units/g, respectively, and were completely inactivated by Ellman's reagent. These findings indicate: (a) that the abnormal adenylate kinase activity found in dystrophic muscles (Fig. 2) is likely to be localized in the muscle fibres, and (b) that it is not present during the final stages of the disease.

Although it is tempting to speculate that an isozyme of the liver type is present in dystrophic muscle (*cf.* Fig. 2) at least one other explanation of our results is possible: the SH groups of the "normal" muscle adenylate kinase could have been modified in the diseased muscle *in vivo* in such a way that the enzyme (a) was still active and (b) could not be inactivated by DTNB because there were no free SH groups. A situation similar to this was produced experimentally[9]; preincubation of human muscle adenylate kinase with methylmercury nitrate led to 25% loss of activity but protected the enzyme against further inactivation by Ellman's reagent; *i.e.* the remaining activity in the presence of methylmercury nitrate as shown in Fig. 1 was not influenced by adding excess DTNB to the incubation mixture.

It is to be hoped that adenylate kinase isozymes from dystrophic muscle can be isolated and characterized. It should be possible then to demonstrate the modification of "local" proteins directly and/or to elucidate the roles of "foreign" isoenzymes in the diseased muscle.

MATERIALS AND METHODS

Samples of human tissues

Muscle specimens (50–200 mg of quadriceps femoris muscle) from three patients aged 4, 6 and 7 years with progressive muscular dystrophy (Duchenne type) were obtained from biopsies which had been necessary for clinical purposes at the Universitätskinderklinik in Freiburg (Germany). The specimens were kept at −25° and did not show any loss of activity for 3 months under these conditions. Normal muscle (also quadriceps femoris muscle) was obtained from a healthy 6-year-old boy from whose leg a foreign body had to be removed. Specimens of human liver were obtained from the Chirurgische Klinik and the Pathologisches Institut, Universität Heidelberg.

Tissue preparation

1 g of tissue was homogenized in 5 ml of 0.1 M Tris–HNO_3 (pH 8.0) with a Bühler homogenizer for 3 min at low speed. After a clearing centrifugation (MSE 18, 6000 rev./min, 5 min, 4°) the supernatant was diluted with 0.1 M Tris–HNO_3 to 10 enzyme units of adenylate kinase per ml.

Chemicals and biologicals

The same compounds and analytical methods as those described previously[11] were used. The molarity of human muscle adenylate kinase solutions was calculated on the basis of a molecular weight of 21 500.

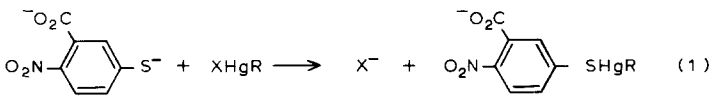
Notes on the measurement of enzymic activity

For most experiments the pH-stat procedure[9,10] was found to be satisfactory. The maximal concentration of DTNB used in the assay mixture was $5 \cdot 10^{-6}$ M and did not interfere with the hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) activity in the coupled enzyme system. When the adenylate kinase activity of crude extracts was tested hexokinase was left out in control assays. As no enzyme activity was observed in the absence of hexokinase it is unlikely that enzymes other than adenylate kinases contributed to the measured activity. Whenever dithiothreitol (2 mM final concentration) was used in the assay mixture it was added before the enzymes hexokinase and adenylate kinase. If a concentrated thiol solution was added rapidly in the presence of the enzymes the activity decreased immediately and unpredictably.

Determination of mercurials

An approx. 5 mM solution of PHMB was made up according to BOYER[15], and a 5 mM solution of methylmercury nitrate in water was a gift from Dr. R. Leberman. A simple procedure was found to be adequate for the determination of the concentrations of mercury in both solutions. This method is also employed for determining

the concentrations of different organic mercurials in solutions used for the preparation of heavy atom derivatives of pork muscle adenylate kinase[16]. The procedure is based on the following reaction (ELLMAN[14]):



2-Nitro-5-thiobenzoate, bright yellow

Mercury derivate of 2-nitro-5-thiobenzoate, colourless unless R is coloured

Preparation of the 2-nitro-5-thiobenzoate reagent

250 μl of Ellman's reagent (10 mM DTNB in 0.1 μ phosphate buffer, pH 7.0) and 25 μl of 14 mM β-mercaptoethanol were mixed and brought to a final volume of 5 ml with 0.25 M Tris–HNO_3 (pH 8.0). This solution has an absorbance of approx. 0.95 at $\lambda = 412$ nm (ref. 14). The solutions for preparing the 2-nitro-5-thiobenzoate reagent are stable for at least one month at 4°; the reagent itself should be used within 2 h.

Determination of a mercurial

2 ml of 2-nitro-5-thiobenzoate reagent were pipetted into each one of a pair of matched 1-cm cuvettes. Then an aliquot of the mercurial solution was added to one cuvette and the same volume of a similar solution without added mercury compound to the other. From the difference in absorbance at $\lambda = 412$ nm the concentration of the mercurial was determined[14] according to the equation:

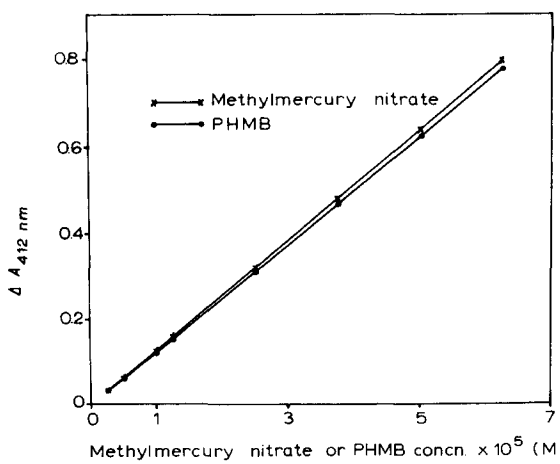


Fig. 3. Calibration curve for the determination of mercurials. 10 pairs of 4-ml samples of 2-nitro-5-thiobenzoate reagent were prepared. From each sample of a given pair an aliquot of V μl was removed and replaced by V μl of a 1 mM mercurial solution in one sample and by V μl of water in the other. The difference in absorbance at $\lambda = 412$ nm between the samples of a given pair was measured after 1 min. The values chosen for V were 10, 20, 40, 50, 75, 100, 120, 150, 200, and 250 μl, respectively. The absorbances at $\lambda = 412$ nm of the samples without mercurial were in the range of 0.96 ($V = 0$ μl) to 0.90 ($V = 250$ μl). These values are, of course, the upper limits for observable differences in absorbance at $\lambda = 412$ nm.

$$c_{\text{mercurial}} = \frac{A_{412\text{ nm}}}{13\,600} \cdot D \qquad (2)$$

where D denotes the dilution factor for the mercurial solution. (The actual concentration of the mercury compound in the cuvette is in the range of $5 \cdot 10^{-6}$–$5 \cdot 10^{-5}$ M, *cf.* Fig. 3.)

The concentrations found by this method were 8% lower than the values found by the method of BOYER[15] for PHMB and 5.5% less than the values determined for methylmercury nitrate by the method of KING[17] which uses pyridine-2-azo-*p*-dimethylaniline[18] as indicator. These differences can be easily accounted for by assuming a value of 12 500 for Ellman's constant for the reaction involving PHMB and a constant of 12 800 in reactions with methylmercury nitrate instead of a value of 13 600 as given in Eqn. 2.

ACKNOWLEDGEMENTS

We thank Prof. R. Beckmann (Universitätskinderklinik Freiburg), chairman of the Gesellschaft zur Bekämpfung der Muskelerkrankungen, who provided us with samples of diseased muscle and supported our work in all respects. We also wish to thank Prof. E. Kuhn and Dr. W. Meinrenken for their critical discussion of the clinical part and Prof. W. Hasselbach and Dr. R. Leberman for discussing the biochemical aspects of our work.

REFERENCES

1 B. T. HOOTON AND D. C. WATTS, *Biochem. J.*, 100 (1966) 637.
2 J. N. WALTON, N. CANAL, G. SCARLATO, *Muscle Diseases, Proc. Int. Congr.*, Excerpta Medica, Amsterdam, 1970, pp. 205–360.
3 R. PALMIERI, H. KEUTEL, H. JACOBS, K. OKABE, R. YUE, F. ZITER, F. TYLER AND S. A. KUBY, *62nd Annu. Meeting, San Francisco, 1971, Fed. Proc.*, 30 (1971) 1255, Abstr. 1181.
4 C. J. NICHOL, *Can. J. Biochem.*, 42 (1964) 243.
5 B. T. HOOTON AND D. C. WATTS, *Clin. Chim. Acta*, 16 (1967) 173.
6 M. CHIGA AND G. W. E. PLAUT, *J. Biol. Chem.*, 235 (1960) 3260.
7 F. S. MARKLAND AND C. L. WADKINS, *J. Biol. Chem.*, 241 (1966) 4124.
8 J. C. KHOO AND P. J. RUSSELL, *62nd Annu. Meeting, San Francisco, 1971, Fed. Proc.*, 30 (1971) 1221, Abstr. 987.
9 L. F. KRESS, V. H. BONO, JR. AND L. NODA, *J. Biol. Chem.*, 241 (1966) 2293.
10 T. A. MAHOWALD, E. A. NOLTMANN AND S. A. KUBY, *J. Biol. Chem.*, 237 (1962) 1535.
11 E. THUMA, R. H. SCHIRMER AND I. SCHIRMER, *Biochim. Biophys. Acta*, 268 (1972) 81.
12 L. F. KRESS AND L. NODA, *J. Biol. Chem.*, 242 (1967) 558.
13 R. H. SCHIRMER, I. SCHIRMER AND L. NODA, *Biochim. Biophys. Acta*, 207 (1970) 165.
14 G. L. ELLMAN, *Arch. Biochem. Biophys.*, 82 (1959) 70.
15 P. D. BOYER, *J. Am. Chem., Soc.* 76 (1954) 4331.
16 I. SCHIRMER, R. H. SCHIRMER, G. E. SCHULZ AND E. THUMA, *FEBS Lett.*, 10 (1970) 333.
17 P. L. KING, Ph. D. Thesis, University of Cambridge, 1971.
18 I. M. KLOTZ AND W. C. LOH MING, *J. Am. Chem. Soc.*, 75 (1953) 4159.