Variability in the ratio of mutant to wildtype myosin heavy chain present in the soleus muscle of patients with familial hypertrophic cardiomyopathy. A new approach for the quantification of mutant to wildtype protein

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Abstract The ratio of mutant to wildtype myosin heavy chain (β-isoform, β-MHC) in the soleus muscle of patients with familial hypertrophic cardiomyopathy was determined by a combination of HPLC, mass spectrometry and capillary zone electrophoresis. In two patients, one with a Val 606 Met mutation and another with a Gly 584 Arg mutation, the fraction of mutant β-MHC was only 12 ± 6% and 23 ± 0.7% of total β-MHC, respectively. These results demonstrate the necessity to determine the ratio of mutant to wildtype protein for the interpretation of functional studies on biopsy material from heterozygous patients with an inherited disease.

Key words: Familiar hypertrophic cardiomyopathy; Missense mutation; Mass spectrometry; β-Myosin heavy chain

1. Introduction

Several inherited diseases are caused by missense mutations that lead to malfunction of the mutant protein. Examples of such diseases are sickle cell anemia, caused by the exchange of a single amino acid in the hemoglobin β-chain [1], or several diseases of the skeletal muscles, caused by missense mutations in genes encoding ion channels [2]. The discovery of mutations in sarcomeric proteins as the cause of familial hypertrophic cardiomyopathy (FHC) extended the list of inherited diseases that are caused by missense mutations. In some families this disease is caused by missense mutations in the β-isoform of the myosin heavy chain (β-MHC) [3,4]. FHC, however, is a very heterogeneous disease. Even members of the same family, i.e. patients with the same mutation, have different clinical symptoms and a very different prognosis [5]. The reason for this variability is still unknown. Since the patients are heterozygous, mutant and wildtype β-MHC are coexpressed. It is, however, not known whether the ratio of mutant to wildtype protein is 1:1. A variation of the ratio of mutant to wildtype protein from patient to patient might explain the wide spectrum of clinical symptoms even within the same family with the identical mutation.

To test this hypothesis, we quantified the ratio of mutant to wildtype protein. This quantification is hampered by the large size of the β-MHC (1935 amino acids) [6,7], because the physico-chemical properties of such a large protein are not sufficiently altered by the exchange of a single amino acid for the mutant and wildtype forms to be separated. This becomes even more difficult when amino acids are exchanged for other amino acids of similar properties.

In the present study, we have developed a new method for the quantification of the ratio of mutant to wildtype protein, which can be applied to large proteins and to any type of amino acid exchange. To use this approach, the amino acid sequence of the protein and the type of mutation must be known. In our strategy, we first digested the large protein with a specific endoproteinase, so that a mixture of well defined peptides was generated. Digestion of the wildtype and the mutant protein generates identical peptides except for the one peptide that includes the position of the point mutation. This peptide exists in two forms, the wildtype peptide and the mutant peptide. Selecting a suitable endoproteinase yields a pair of mutant and wildtype peptides with sufficiently different physico-chemical properties for successful separation.

Because the digestion of a large protein generates a very complex mixture of numerous peptides, a two-step separation is used to eventually isolate pure mutant and wildtype peptides. First, the digest is fractionated by HPLC. Second, each fraction of the HPLC run, still containing a mixture of peptides, is searched for the mutant and the wildtype peptide by mass spectrometry (MS). This search by MS involves two steps, first determination of the molecular weight to identify candidate peptides, and second amino acid sequencing of the candidate peptides which are still in the peptide mixtures [8], which would not be possible with Edman sequencing.

Since MS does not allow quantitation of the amount of peptides present in the starting material, the fractions of the first HPLC run that contained wildtype and mutant peptide respectively were further separated by capillary zone electrophoresis (CZE) to achieve baseline separation for quantification of the wildtype and the mutant peptide from the peak areas. Relevant peaks were identified with synthetic peptides that served both as markers and as standards.

We applied this method to two different missense mutations in the β-MHC causing FHC. In one patient, Val is replaced by Met at position 606 (Val 606 Met mutation), and a Gly 584 Arg mutation was found in the other patient. In this study, we took advantage of the fact that the β-MHC is expressed not only in the ventricle but also in slow skeletal muscles like the soleus muscle.

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2. Materials and methods

2.1. Preparation of myosin

Fiber bundles (1–3 mm diameter, 5–10 mm length) from the soleus muscle were obtained from patients with FHC and healthy controls. These bundles were prepared as previously described [9–11] and stored in liquid nitrogen [12]. While still frozen, each bundle was homogenized and myosin was extracted with 150 μl of a modified Hasselbach-Schneider solution (0.5 M NaCl, 10 mM HEPES, 5 mM $MgCl_2$, 2.5 mM ATP; potassium in the original Hasselbach-Schneider solution was replaced by sodium) for 15 min at 4°C. After centrifugation, the supernatant was diluted with 10 volumes of water and myosin was allowed to precipitate at 4°C for 24 h. The supernatant was discarded and the precipitate resuspended in 100 μl 0.1% trifluoroacetic acid (TFA) and then lyophilized.

2.2. Digestion of myosin by endoproteinase Lys-C

Lyophilized myosin was digested with 5 μg endoproteinase Lys-C (Boehringer Mannheim, Germany) in 0.1 M NH_4HCO_3 for 24 h at 37°C. The digest was lyophilized.

2.3. HPLC separation of peptides

The lyophilized peptides were dissolved in 50 μl 0.1% TFA and chromatography was carried out using a 2 mm×250 mm C18 reversed phase column (Reprosil C18, 5 μm, 12 nm, Dr. Maisch, Feldbuch, Germany) at a flow rate of 0.2 ml/min. The column temperature was maintained at 30°C. A linear gradient using 0.1% (v/v) TFA in water (solvent A) and 0.1% TFA in 40% methanol, 40% 2-propanol in water (solvent B) was generated, starting with 10% of solvent B increasing to 70% of solvent B within 60 min. UV absorbance was monitored at 220 and 280 nm. Fractions were collected every minute.

2.4. Mass spectrometry

Mass spectrometric analysis was carried out with an API III triple stage quadrupole mass spectrometer (PE-SCIEX, Langen, Germany), equipped with articulated ion spray. The HPLC fractions were either used directly for the mass spectrometric analysis or lyophilized and then redissolved in 0.5% formic acid, 50% acetonitrile in water (v/v). Solutions were infused at a flow rate of 5 μl/min, using a syringe pump.

Molecular weight was determined in the positive ion mode in a mass/charge (*m/z*) range from 400 to 2400. All settings of the instrument were optimized each day during the calibration procedure. For mass spectrometric sequencing of peptides by MS/MS, we used argon as collision gas. Mass spectra were evaluated with the MacSpec (PE-SCIEX, Langen, Germany) and SHERPA [13] programs.

2.5. Capillary zone electrophoresis

CZE was performed with an uncoated 50 μm×50 cm fused silica capillary. The separation buffer was 100 mM sodium phosphate, pH 3.0, with 0.02% hydroxymethylcellulose. The peptides were separated at 25°C with a constant current of 80 μA, UV absorbance was recorded at 200 nm. The sample was applied with low pressure at a flow rate of about 6 nl/min. A standard peptide (Low pH Mobility Standard, PE-Applied Biosystems) was added to each sample to control the sample volume that was applied.

Synthetic peptides were used for the identification of peaks in the CZE. These peptides were synthesized with the FMOC strategy, using a 430 A peptide synthesizer (PE-Applied Biosystems).

2.6. SDS-PAGE

SDS-PAGE was carried out using the PhastSystem (Pharmacia Biotech). 12.5% Phastgels were used and the sample buffer was 10 mM Tris-HCl, 1 mM EDTA, 2.5% SDS, 5.0% 2-mercaptoethanol and 0.01% bromophenol blue. Gels were stained with PhastGel Blue R (Pharmacia Biotech).

3. Results

The general procedure is illustrated in the flow chart in Fig. 1.

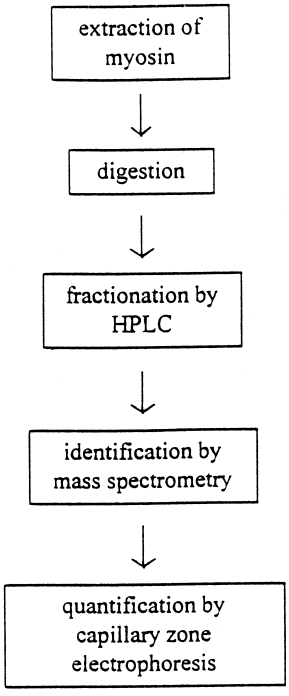

Fig. 1. Flow chart showing the general approach for the quantification of the ratio of mutant to wildtype protein.

3.1. Isolation of myosin from fiber bundles

Myosin was isolated from soleus muscle tissue by high salt extraction and subsequent precipitation. The purity of the myosin was checked by SDS-PAGE (Fig. 2). No impurity was detectable by staining the gel with Coomassie brilliant blue. We found this degree of purity to be sufficient for further analysis of the mutant and wildtype protein. Whole myosin, i.e. heavy chains and light chains, was isolated by this high salt extraction. Only 50–70% of the total myosin was isolated by this method. The yield increased with increasing time of extraction, but contamination with other proteins also increased. Therefore, we preferred an extraction time of 15 min, despite the incomplete extraction.

3.2. Digestion of myosin and HPLC separation

For digestion we used the endoproteinase Lys-C. This enzyme cleaves the peptide bond very specifically at the carboxylic site of lysine. From the sequence of the β-MHC, it is expected that digestion by endoproteinase Lys-C generates more than 200 peptides ranging in length from 3 to 37 amino acids. The peptide including position 606 is 13 amino acids long. The wildtype peptide has a molecular weight of 1474.5, and the mutant peptide 1506.5. The difference equals the mass difference between Val and Met. Unexpectedly large fragments, e.g. resulting from incomplete digestion, were not found by MS.

The endoproteinase Lys-C digest was first fractionated by HPLC. A typical chromatogram is shown in Fig. 3. There is a large number of overlapping peaks, i.e. the resolution is poor, due to the large number of peptides generated by the digest. Since the total number of peaks is much smaller than 200, it is expected that individual peaks still contain several different peptides. The limited resolution makes a quantitative analysis of individual peptides impossible. Therefore the fractions con-

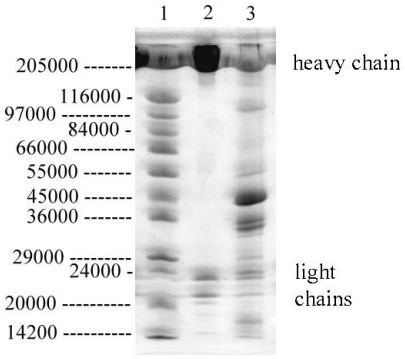

Fig. 2. SDS-PAGE. Lane 1: molecular weight marker; lane 2: purified myosin after extraction and precipitation; lane 3: remaining muscle tissue after extraction of myosin. Note that the heavy chain and the light chains were extracted. The muscle tissue still contained some myosin after extraction.

taining wildtype and mutant peptide had first to be identified by MS and then further separated by CZE.

3.3. Mass spectrometry

To identify the HPLC fractions that contain wildtype and mutant peptide, each HPLC fraction was analyzed by ESI-MS. Fig. 4a shows a mass spectrum of one HPLC fraction in which two cations with an *m/z* of 1476.1 and 738.5 were detected, corresponding to the *m/z* ratio of a single and a double positively charged peptide, respectively, i.e. with *m/z* ratios expected for the wildtype peptide. In a second HPLC fraction (Fig. 4b), a cation with *m/z* of 1507.5 was found, corresponding to the *m/z* expected for a single charged peptide with the mass of the mutant peptide. In a control experiment with muscle tissue from a healthy individual, we could only detect the wildtype peptide, while the second fraction did not show the peak seen for the mutant peptide.

To confirm that the identified ions represent ions originating from wildtype and mutant peptides, we determined their amino acid sequences by mass spectrometry (MS/MS). Sequencing of peptides by MS/MS, using a triple quadrupole mass spectrometer [8], is achieved as follows: the ion of interest (i.e. the charged peptide) is selected by its specific *m/z* value in the first quadrupole chamber, and other ions are discarded. This selected peptide (called parent ion) is dissociated into fragments (daughter ions) by collision with argon atoms in the second chamber. These fragments are separated and detected in the third quadrupole chamber. In the fragmentation process, mainly the peptide bond is cleaved and the charge remains associated with one of the two fragments. When the N-terminal fragment is charged, it is called a b-ion, and when the C-terminal fragment is charged, it is called a y-ion. Defined by the *m/z* ratio, two series of daughter ions are generated by the fragmentation of the parent ions, a b-series and a y-series. The differences in the molecular weight between nearest neighbor ions of one series correspond to the molecular weight of one amino acid and so the sequence of the peptide can be reconstructed from the series of daughter ions. The sequencing spectra of the wildtype and mutant peptide are shown in Fig. 5. Note the mass (*m/z* for $z = 1$) difference between daughter ions y5 and y6 in the sequencing spectra of the wildtype and the mutant peptide. This difference is caused by the different molecular weights of Val and Met. Thus, MS/MS analysis allowed us to identify wildtype and mutant peptide by their amino acid sequence without isolating pure peptides from the original HPLC fraction [8].

The soleus muscle contains not only the β-isoform but also various other isoforms of the myosin heavy chain. The composition of these isoforms is variable depending on training, humoral and disease status. Therefore the identification of peptides from the β-isoform by sequencing is necessary to avoid errors originating from the detection of peptides from other isoforms. Since the amino acid sequences of the peptides used for the quantification of the Val 606 Met and the Gly 584 Arg mutation are unique for the β-isoform as found by comparison with all known amino acid sequences of human MHC, other isoforms do not interfere with the quantification of the ratio of mutant to wildtype β-MHC.

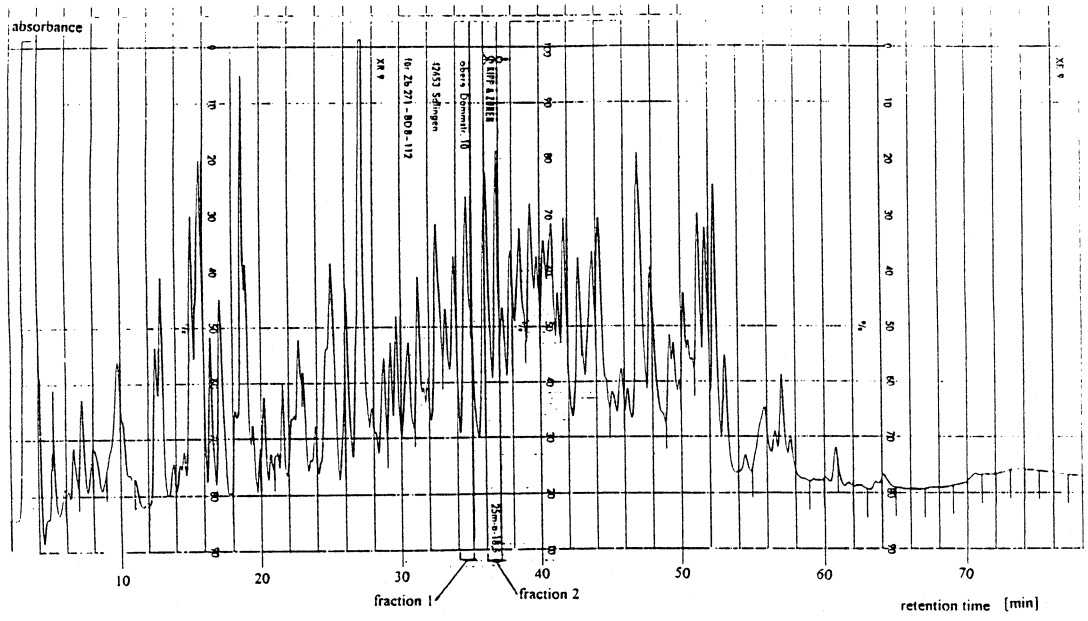

Fig. 3. HPLC chromatogram of the mixture of peptides generated by the digestion of myosin by endoproteinase Lys-C. Fraction 1 contains the wildtype peptide, fraction 2 the mutant peptide.

3.4. Capillary zone electrophoresis

Since there are no suitable standards, MS with peptides does not allow quantitation of the amount of peptides in the starting material [14], i.e. the peak area cannot be directly used to estimate the amount of peptide in the HPLC fraction applied to the mass spectrometer. Thus, for quantification we used CZE to achieve baseline separation of the peptides contained in the two HPLC fractions that include wildtype or mutant peptide, such that both peptides could be quantified from their corresponding peak areas. Fig. 6 shows the electropherograms. Note that wildtype and mutant peptide have the same migration time. This means that they could not have been separated if they had been present within the same HPLC fraction. Thus, successful separation into two different fractions by the initial HPLC run was essential for the separation and quantification of wildtype and mutant peptides. In control runs, synthetic wildtype and mutant peptides were used to identify the relevant peaks in the CZE runs, and to calibrate the peak areas. The ratio of mutant to wildtype peptide was evaluated from the corresponding peak areas. Analyzing five fiber bundles from one patient with the Val 606 Met mutation with this protocol, we found only $12 \pm 6\%$ of total β-MHC as mutant heavy chain.

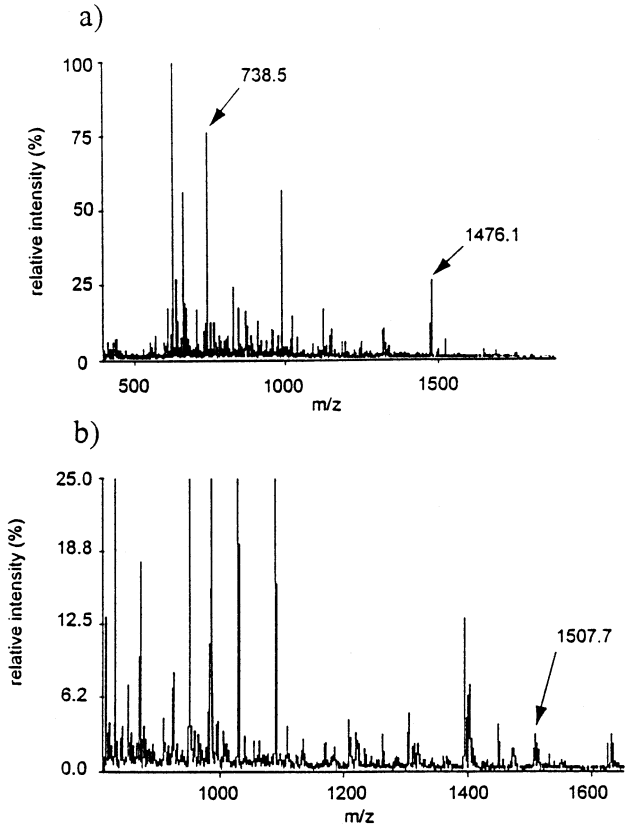

Fig. 4. a: Mass spectrum of the HPLC fraction containing the wildtype peptide. The peaks of the single charged peptide ($m/z = 1476.1$) and the double charged peptide ($m/z = 738.5$) are labeled by arrows. The intensity of the peaks is normalized to the highest peak. b: Mass spectrum of the HPLC fraction containing the mutant peptide. The peak of the single charged peptide ($m/z = 1507.7$) is labeled by an arrow. In contrast to the wildtype peptide, the double charged mutant peptide could not be detected. The highest peak ($= 100\%$) is not shown in this spectrum.

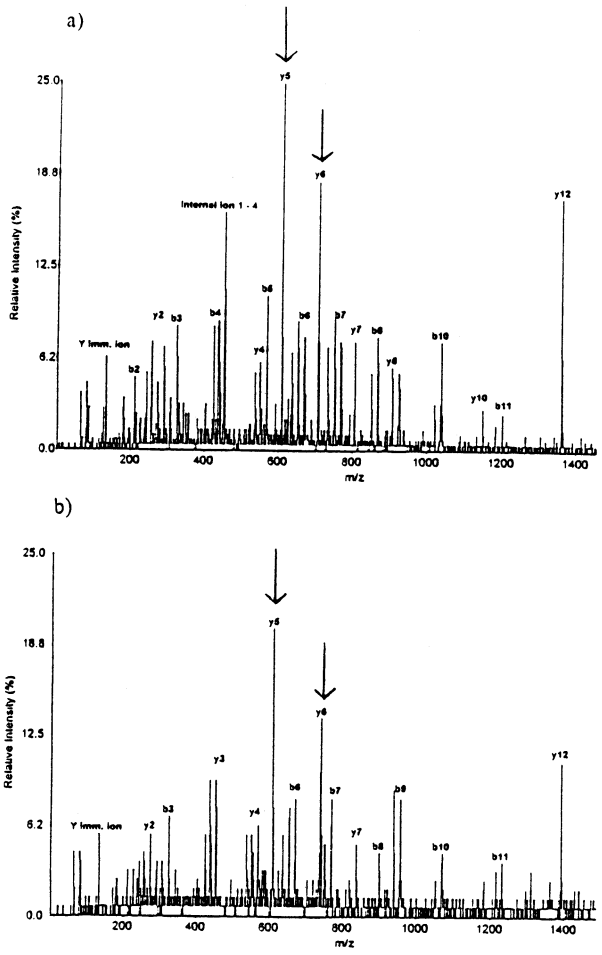

Fig. 5. Sequencing spectra of the wildtype peptide (a) and the mutant peptide (b). The mass difference between ions y5 and y6 (labeled with arrows) corresponds to valine in a and to methionine in b, showing the Val 606 Met mutation. This analysis was carried out with HPLC fractions of the endoproteinase Lys-C digest of myosin, which were further analyzed by CZE.

3.5. Controls

To exclude systematic errors in the analytical procedure, we carried out several control experiments. (i) Synthetic peptides were used to demonstrate that wildtype and mutant peptide have the same UV absorbance. (ii) It was further demonstrated that the peak area in the electropherogram is a linear function of the amount of applied peptide. (iii) Every sample analyzed by CZE contained a known amount of a synthetic standard peptide (Low pH Mobility Standard, PE-Applied Biosystems) to correct fluctuations in the sample application and increasing concentrations of peptides in the samples due to evaporation of solvent prior to loading on the CZE. The migration time of this standard peptide was different from the mutant and the wildtype peptide.

(iv) Detectable loss of a mutant or wildtype fragment in the analytical procedure was excluded by the following control experiment: the digest of β-MHC from a healthy individual was divided into two equal parts, and known amounts of synthetic peptides (wildtype and mutant) were added to one part. The whole analytical procedure was carried out with both parts. To estimate the detected amount of synthetic wildtype and mutant peptides, the corresponding peak areas of the

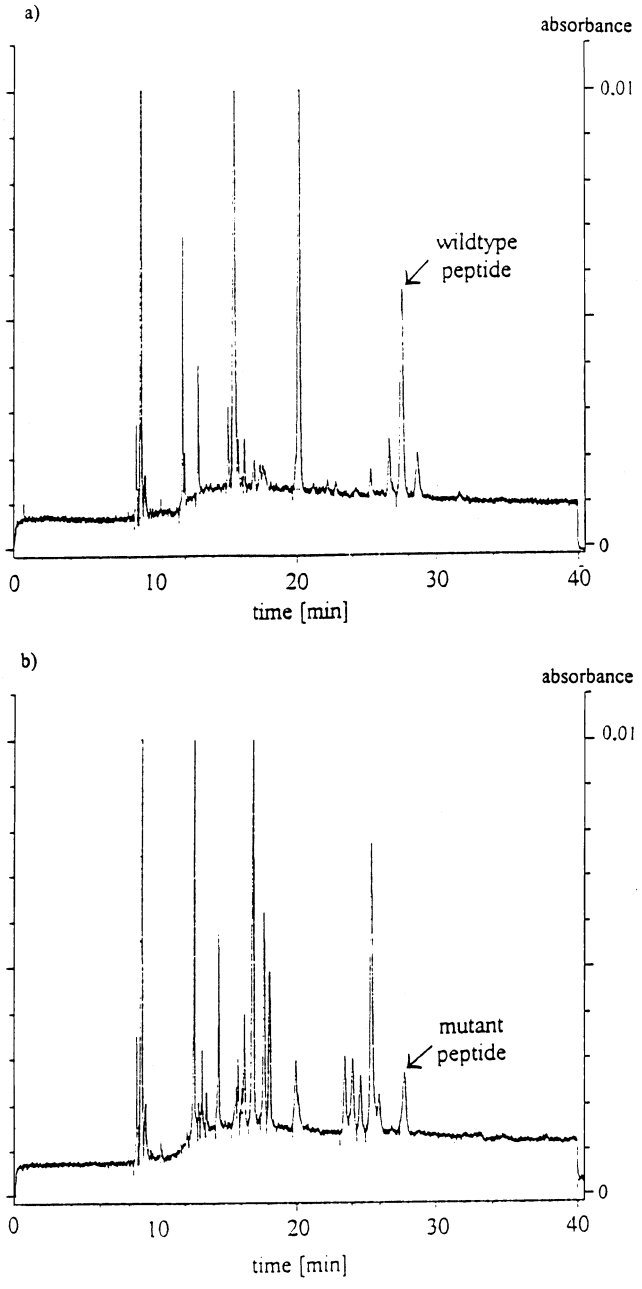

Fig. 6. Electropherograms of the HPLC fraction containing the wildtype peptide (a) and the HPLC fraction containing the mutant peptide (b). Both HPLC fractions are a mixture of peptides that are separated by CZE. In both electropherograms shown here, the peak with the lowest migration time is a standard peptide (Low pH Mobility Standard, PE-Applied Biosystems).

sample without synthetic peptides were subtracted from the total peak areas of the sample with the synthetic peptides. For both peptides (wildtype and mutant), the remaining peak area, i.e. the amount of peptide detected in the CZE run, was essentially identical to the amount initially added to the digest, meaning there was no loss, specifically no unequal loss, of wildtype and mutant peptide.

(v) To estimate errors in the quantification, we carried out two further controls; first we ran the HPLC and subsequent CZE analysis with synthetic peptides under exactly the same conditions as with the digests. Running five test samples, we found a standard error of ±12% of the observed mean value. Analyzing one sample of a synthetic peptide four times with CZE, the standard error was ±4% of the observed mean value.

Once the specific procedure had been established (preparation of synthetic peptides, identification of the relevant fractions of the HPLC separation step, etc.), quantification of mutant to wildtype protein content of a tissue sample required some 3 days, mainly due to the time required for precipitation of extracted myosin and its digestion.

3.6. Analysis of the Gly 584 Arg mutation

We applied the same procedure to β-MHC with a Gly 584 Arg mutation. One remarkable difference to the Val 606 Met mutation was that wildtype and mutant peptide eluted together in the same fraction in the HPLC run. These peptides, however, had different migration times in the CZE so that they were separated at this step. This illustrates that separation of wildtype and mutant peptide by the initial HPLC run is not essential if they can be separated by CZE. Here, the HPLC run only serves for an initial fractionation of the large number of peptides. Analysis of four fiber bundles from one patient with the Gly 584 Arg mutation yielded a fraction of mutant β-MHC of 23 ± 0.7% of total β-MHC. For the Gly 584 Arg mutation, we also found that there was no loss of wildtype or mutant peptide. This was tested again using synthetic peptides as described in Section 3.5.

4. Discussion

The aim of our study was to develop a generally applicable procedure that allows quantification of the ratio of mutant to wildtype protein for large molecules, whose physico-chemical properties are not sufficiently changed by a single amino acid exchange such that the mutant and the wildtype protein can easily be separated by standard methods. We used β-MHC as an example of such a large protein and demonstrated the potential of our approach by analyzing two mutations, Val 606 Met and Gly 584 Arg. The Val 606 Met mutation is a particularly difficult example since both amino acids have very similar properties. We succeeded using a combination of enzymatic digestion, HPLC, MS and CZE. Previously, Cuda et al. [15] used an approach based on peptide mapping and Western blot analysis. However, this method has two disadvantages. (i) The protein has to be cleaved right at the point of the mutation. For some mutations, there is no appropriate enzymatic or chemical digest. (ii) For each mutation, a specific anti-peptide antibody has to be raised. Some amino acid sequences, however, are only poor antigens, which is presumably the reason for our failure when trying to apply this method to the Val 606 Met and the Gly 584 Arg mutations.

We therefore attempted to establish an approach which can generally be applied for the quantification of the ratio of mutant to wildtype protein, independent of the type of protein and of the type of amino acid exchange. The approach presented here only fails for the exchange of Leu to Ile, because of their identical molecular weights. With this new method, we were able to show that in the soleus muscle from two patients with FHC the fraction of mutant β-MHC is only 12 ± 6% (Val 606 Met mutation) and 23 ± 0.7% (Gly 584 Arg mutation) of total β-MHC. In contrast to the Gly 584 Arg mutation, the analysis of the Val 606 Met mutation has a

large standard error (±50%), although both mutations were analyzed by the same procedure. One explanation for the different standard errors may be differences in the starting material, for instance a large variation in the ratio of mutant to wildtype protein among different fiber bundles within the biopsy with the Val 606 Met mutation, whereas the biopsy material with the Gly 584 Arg mutation may have been more homogeneous. In addition, the small standard deviation might result from the fact that both wildtype and mutant fragments eluted in the same HPLC fraction. The two fragments were therefore analyzed within one CZE run, i.e., the corresponding peak areas could directly be related without the need of a standard peptide for calibration.

There are several requirements which must be met for a reliable determination of the ratio of mutant to wildtype protein. The HPLC fractions with wildtype and mutant peptide have to be identified as well as the corresponding peaks in the CZE. We found sequencing by mass spectrometry and the use of synthetic peptides as standards in the CZE to be suitable to meet these goals. The synthetic peptides were also indispensable tools to show that (a) wildtype and mutant peptide had the same UV absorbance in the CZE, (b) the peak area is proportional to the amount of peptide and (c) the recovery of both peptides is identical. The last point is of particular importance because different peptides may, for instance, adsorb to the surfaces of reaction vessels to a different extent, so that their recovery in the analytical procedure might differ.

Another source of error could be the differential extraction of the wildtype and mutant myosins in the first step of the procedure. This, however, is unlikely, because mutations in the globular head domain are not expected to have much effect on attractive forces between the tail domains of myosin molecules that form the myosin filaments. In addition, the exchange of only one amino acid in such a large protein has only little effect on physico-chemical properties like solubility.

Due to the complexity of the mixture of peptides resulting from the digestion of a large protein, two separation techniques are necessary to achieve sufficient resolution for quantification of the peptides. As we have shown in our analysis, it is not important whether the wildtype and the mutant peptide are separated in the first or the second step (HPLC or CZE). We used the combination of RP-HPLC and CZE, but the combination of other separation techniques may also be possible, if they have different physico-chemical principles of separation. The combination of different separation methods extends the range of proteins that can be analyzed with our method. In addition, the use of enzymatic or chemical digests other than the endoproteinase Lys-C digest makes the method even more versatile. While in the approach of Cuda et al. [15], cleavage has to occur right at the point of mutation, our method only requires that cleavage generates a peptide that includes the mutated position and which is small enough that the mutation changes its physico-chemical properties sufficiently for separation. Therefore, one can choose from a wide variety of specific endoproteinases or a specific chemical cleavage.

In contrast to our results, Malinchik et al. [16] reported the presence of equal amounts of mutant and wildtype β-MHC in the soleus muscle of a patient with FHC caused by an Arg 403 Gln mutation. Taken together with the data presented here, this shows that the ratio of mutant to wildtype β-MHC can vary greatly, at least for different mutations. Differences in the ratio of mutant to wildtype β-MHC may be caused by differences in the transcription of the mutant gene, differences in the translation or in the lifetime of the mutant mRNA or differences in the incorporation of the mutant protein into the contractile apparatus. Since the mutations are discovered by single-strand conformation polymorphism, a different conformation of the mutant mRNA is possible. Therefore, the translation may be impaired or the lifetime of the mutant mRNA may be shorter, so that there is less mutant than wildtype mRNA. That a decreased translation of mutant mRNA, however, is also a possible reason for unequal amounts of wildtype and mutant protein was demonstrated by Choong et al. [17]. These authors reported that, in COS cells, expression of the human androgen receptor with a missense mutation is reduced compared to the wildtype androgen receptor, while the amounts of mutant and wildtype mRNA were equal. With myosin binding protein C Rottbauer et al. [18] showed the presence of the mutant mRNA but the virtual absence of the corresponding protein. But there is also evidence from transgenic animals that the mutant mRNAs are translated [19,20], in some cases even in relatively high levels. Some results suggest that changes in protein stoichiometry may be relevant for the degree of myofibril disorder in sarcomeric protein mutations [21]. This may also be the case with missense mutations in the myosin heavy chain causing FHC.

Due to the small fraction of mutant β-MHC found in this study, while Malinchik et al. [16] found 50% for the Arg 403 Gln mutation, the interpretation of functional studies on biopsy material from patients with FHC requires quantification of the mutant protein. Otherwise, differences in functional impairment may be mistaken for specific effects of the different mutations, while they may simply result from differences in the presence of mutant protein.

As starting material we used fiber bundles which were treated with a skinning solution, so that all soluble proteins were lost. Only the myosin integrated into the sarcomeric structure was analyzed. This is advantageous, because the detection of soluble mutant myosin that is not integrated into the contractile apparatus would interfere with the correlation between the ratio of mutant to wildtype protein and the functional effects of the mutation; functional studies are being carried out with skinned fibers since this system allows for a much larger spectrum of functional tests than possible with native fibers.

The reasons for the wide variety of clinical symptoms in FHC, even within the same family, are still unknown. This variation may perhaps result from differences in the presence of mutant β-MHC. With the approach described here, this hypothesis can now be tested by analyzing biopsies from different members of one family. Another interesting question is whether the fraction of mutant protein changes with age. This idea is supported by the observation of Geisterfer-Lowrance et al. [22], who described a mouse model for FHC. In these mice, the clinical symptoms of FHC became more severe with age, and such changes could again be caused by a change in the ratio of mutant to wildtype protein. The method presented here for determination of this ratio now allows such questions to be addressed.

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