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Review

Separation and identification of human heart proteins

D. Jäger[a,*], P.R. Jungblut[b], U. Müller-Werdan[a]

[a]*Department of Medicine III, Martin-Luther University, Halle-Wittenberg, Germany*
[b]*Max-Planck Institute of Infection Biology, Berlin, Germany*

Abstract

Heart failure is not a uniform disease entity, but a syndrome with various causes, including hypertension, ischemia and congenital heart disease, cardiomyopathy, myocarditis and intoxication. During the recent years a number of molecular and cellular alterations have been identified in the diseased heart, but a direct causative link between these changes and functional impairment, medical responsiveness, progression of the disease and the patients' outcome remains to be established. After an accumulation of large amounts of DNA sequence data in genomic projects, scientists have now turned their attention to the central executors of all programs of life, the proteins. In complementation of the genomic initiatives, proteomics based approaches have lined up not only for large-scale identification of proteins and their post-translational modifications, but also to study the function of protein complexes, protein–protein interactions and regulatory and signalling cascades in the cellular network. In concert with genomic data functional proteomics will hold the key for a better understanding and therapeutical management of cardiovascular diseases in the future.

Keywords: Reviews; Proteomics; Dilated cardiomyopathy; Human heart proteins; Eucaryotic elongation factor-2

Contents

*Corresponding author. Tel.: +49-345-557-4545; fax: +49-345-557-4546.
E-mail address: doris.jaeger@medizin.uni-halle.de (D. Jäger).

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1. Introduction

1.1. From genomics to proteomics

The basis for proteomic investigations of the human heart [1,2] was set long before the completion of the nucleotide sequence of the human genome [3]. Now the fundamental work on the functional macromolecules important for the understanding of life is gaining more interest. Direct comparison of transcriptomic data with the corresponding proteome data showed clear differences between the transcriptome and the proteome [4,5]. This is obvious from theory, because the transcriptome mainly contains genomic information, whereas the proteome is to a much greater extent imprinted by environmental influences. The genome is, relative to the proteome, more static, and the impact of the environment increases from genome over transcriptome to proteome. Within the proteome it depends on the visualization method, if genetic or environmental influences are to be analyzed. Radioactive labelling allows the view on the protein synthesis situation, where the environmental influences are reduced, roughly representing protein expression. Staining of protein patterns from two-dimensional gels detects the composition of all present proteins, independently from time of their synthesis and including all modifications during their lifetime. Because of modified proteins arising from alternative splicing of transcripts, RNA editing or extensive posttranslational processing [6,7], it is apparent that the paradigm of one gene encoding one protein is no longer tenable. Therefore the functional complexity of an organism can only be dissected and understood when genomic and proteomic tools are used in concert [6–9]. For example it has been shown that one protein may be modified into up to 59 protein species as exemplified for Hsp27 in human heart [10]. The term protein species was defined as the smallest unit of the proteome by its chemical structure [11]. Therefore, a one-fold and a two-fold phosphorylated myosin light chain I are two different protein species of the protein MLCI.

The genome of an individual can be completely sequenced. Meanwhile it was shown that proteomics can help to confirm and even complete the inventory of genes obtained by open reading frame prediction programs [12]. The proteome of an individual can never be complete, because it is time-dependent, and this leads to a continuous change during lifetime, resulting in an infinite number of proteomes per individual. To detect disease-associated protein species by comparison of tissue samples it is therefore necessary to compare many independent sample preparations of many individuals to have the chance to detect proteomic signatures as defined by van Bogelen et al. [13]. Proteomics may provide us with information never before imaginable, but proper design of the study and excellent bioinformatic tools are paramount [7].

The milestones for the meanwhile classical approach of proteomics were the development of 2-DE [14,15], protein sequencing [16], mass spectrometry (MS) using peptide mass fingerprinting [17] and sequence tags [18]. Up to 10 000 protein species can be resolved by high-resolution 2-DE [19] with a sensitivity of some molecules per cell using radiolabelling, or of ng protein amounts detected by silver staining. These tools are prerequisites for a hypothesis-free approach to biological questions. On the basis of an inventory of molecules on the protein level (e.g. heart proteins) [20,21] and the mRNA level [22,23], the apparent information gap between

the genome and the cellular processes in heart physiology and pathophysiology might be closed [24].

In 1995 the term proteome was defined [25], and the promise that proteomics can give insight into the functioning of a cell now is fascinating the life science community. New proteomic techniques are arising continuously, like immunoproteomics [26], molecular scanner to automate proteomic research [27], multidimensional chromatography/MS/MS coupling [28,29], chip technologies [30] using affinity materials [31], antigens [32,33], antibodies or aptamers [34–37], subfractionation in organelles and complexes [38–41] and two-hybrid systems [42,43]. The multidimensional chromatography/MS/MS approach aims at a complete resolution of a proteome, and in its combination with isotope-coded affinity tags [44] it is able to resolve proteins not detected on 2-DE gels with the possibility of exact quantification.

A tendency to introduce all protein analysis techniques under the term proteomics can be seen in many publications, although often only one protein is purified and/or its secondary and tertiary structure or other characteristics are determined. Of course all protein analytical methods contribute to the knowledge about proteins, but one should clearly distinguish between hypothesis-free and reductionistic approaches. Even chip technologies reduce the view on known components immobilized on the chip. Only if all protein species of a proteome were known, an ideal chip containing all corresponding antibodies could be constructed. In addition to thousands of genes about 500 posttranslational modifications are known. Therefore this possibility seems unfeasible.

The heart with its relatively uniform tissue is a clearly defined organ and therefore predestined as a starting material for proteomics with the aim to understand the molecular functioning of a complete organ. This idea led to the first heart databases [1,2,45–47], even at a time of low popularity of proteomics, to several original publications ([48–51] and many others) reviewed here and even some reviews [7,8,24,52] updated here.

Organic chemistry and combinatorial chemistry produced an overwhelming number of chemical compounds, which now can be used in a discovery approach to look for targets within proteomes with a not less overwhelming number of protein species. The understanding of pathogenesis and drug discovery now are at the beginning of a new era in life sciences.

2. Methods of proteomics

The classical proteomic approach, independent of sample origin, comprises sample acquisition and preparation, separation of protein species using two-dimensional gel electrophoresis (2-DE), imaging, identification of protein species and possibly their posttranslational modifications (PTMs), subsequent quantification, which allows comparative studies, and finally data evaluation including the involvement of www-accessible databases. Within each step of this proteomic analysis a number of alternative or additional options exist, and they are continuously becoming more sophisticated. The advances and perspectives in the field of proteomics in general and of cardiovascular proteomics were recently comprehensively reviewed by Macri et al. [24], Arrell et al. [7], Dunn et al. [8] and Banks and coworkers [6].

2.1. Sample preparation

An effective sample preparation constitutes the basis of a successful proteomics bound study. Artifacts introduced at this point of the analysis can often be magnified and potentially jeopardize the validity of the results [24]. The excellent resolving power of 2-DE is a ‘dual-edged sword’ with the ability to detect subtle posttranslational modifications as well as artifactitious modifications such as protein carbamylation due to excessive sample heating or sonification [53].

The ideal sample preparation buffer would be able to completely solubilize all proteins of a tissue/cell preparation or cellular compartment (‘subproteomics’, see below) without adding modifications [24]. The constraints for a good solubilization medium for 2-DE are therefore to be able to break ionic bonds, hydrogen bonds, hydrophobic interactions and disulfide bridges under conditions compatible with isoelectric focusing (IEF), i.e. with very low amounts of salt and other charged compounds (review in [54]).

The homogenization (mechanical, sonification) of a tissue/cells should, if possible, already be performed in a suitable 'lysis buffer', which often includes the use of a protease inhibitor cocktail to inhibit the inherent proteolytic activity associated with the sample. The disrupture of the numerous protein–protein and protein/non-protein interactions is accomplished by the application of various combinations of reducing agents (e.g. dithiothreitol (DTT)), chaotropic agents (e.g. urea or combination of urea and thiourea) and detergents [54]. There have been a number of comprehensive reviews regarding the preparation and utilization of protein 'solubilization buffers' for 2-DE [24,53–55]. Due to the incompatibility of ionic detergents such as SDS with IEF, non-ionic detergents like Triton X-100 or Nonidet P-40 and zwitterionic detergents like CHAPS are routinely preferred [24]. An improved protein solubility has been reported if replacing DTT by phosphine derivatives like tributylphosphine or tris (carboxyethyl) phosphine as reducing agents [54,56]. Moreover, increasing the pH to 10.0 or 11.0 per se will also promote solubilization [54]. The most important problem linked with the chaotrope urea is carbamylation of proteins, which leads to artifactual charge heterogeneity possibly giving rise to the misinterpretation as PTMs. This can be counteracted by the use of pure grade urea, avoidance of temperatures above 37 °C and addition of a cyanate scavenger like carrier ampholytes [54].

2.2. Sample prefractionation (subproteomics)

Besides the analysis of the proteome of a *whole-cell homogenate* it is often preferable to prepare a set of subproteomes as a means of concentrating proteins which may be under-represented within the total protein profile [56]. The need for prefractionation has been recognized very early and has gained attraction again after definition of the term proteome [57]. Fractionation methods exploiting specific protein characteristics can focus on enrichment of a single protein or group of proteins [58], with selectivity based on physicochemical properties, or on differential cellular compartmentalization [7]. An alternative method to increase the presence of low level proteins is to peel away individual layers of the proteome which simplifies this needle-in-a-haystack task [59], e.g. by removal of albumin or the immunoglobulin fraction from serum [60,61]. The sequential use of different detergents has equally been applied to extract specific protein fractions [62], as well as affinity-based methods [38], immunoprecipitation and concanavalin A [63].

Membrane-associated proteins pose a particular challenge with respect to solubilization, and they are significantly underrepresented in 2-D gels. The extraction of membrane proteins from the pellet of the cytosolic fraction can be achieved by sequential solubilization using a combination of urea, thiourea, tributyl phosphine and multiple zwitterionic surfactants [62]. In addition to this the recently described alternative zwitterionic detergent alkylaryl aminosulfobetaine C8psi was utilized in an attempt to facilitate the solubilization of cardiac *sarcoplasmic* and *sarcolemmal proteins* like phospholamban, Ca^{2+}-ATPase and calsequestrin [64,65]. Recent developments concerning the solubilization of (hydrophobic) membrane proteins have been thoroughly reviewed by M.P. Molloy [66] and V. Santoni [67]. The current methodology to isolate membrane proteins has become more and more specific until the total absence of contaminating cytosolic proteins was realized [68].

Other subproteomic approaches focus on *Golgi apparatus membranes* [69] or profiling of the entire rat *mitochondrial proteome* using affinity fractionation and automation [38].

M. Mann and coworkers have added to the subproteomic knowledge by elucidating the spatial organization of *multi-protein complexes*, which perform most cellular functions. They first purify components of a protein complex via molecular interactions using an affinity tagged member. Then the partially cross-linked purified complex is analyzed by mass spectrometry at the femtomole level [39]. The first entire mammalian multi-protein complex, which was excised and characterized from a 2-DE gel, was the the ribosome of *E. coli* long before the term proteome was defined [70].

Other cellular compartments that have been isolated and subject to subproteomics are the *spliceosome*, the machinery that removes introns from mRNA precursors [40], *myofilament proteins* of the heart [59], the *phagosome* [41], *nuclear proteins*

[71–73], *endosomes* [74], *mitochondria* [75] and the *mitochondrial ribosome* [76].

2.3. Protein separation

2.3.1. Two-dimensional SDS polyacrylamide gel electrophoresis (SDS–PAGE)

It is generally agreed that at the present time 2-DE remains unsurpassed in its ability to resolve large protein mixtures. One can routinely separate 2000–3000 protein species from a single sample preparation, or up to 10 000 protein species from large-scale (23×30 cm) gels (Fig. 1) [11,19,45,77]. During the first dimension, or isoelectric focusing stage, proteins are separated via a pH gradient and allowed to migrate to their respective isoelectric points (p*I*) in the gel on the basis of their charge [78]. Proteins are then resolved orthogonally according to their relative molecular mass (M_r) in the second dimension, normally by SDS–PAGE [7]. Technical aspects of 2-DE have been extensively reviewed [79–81]. Polyacrylamide tube or rod gels have traditionally

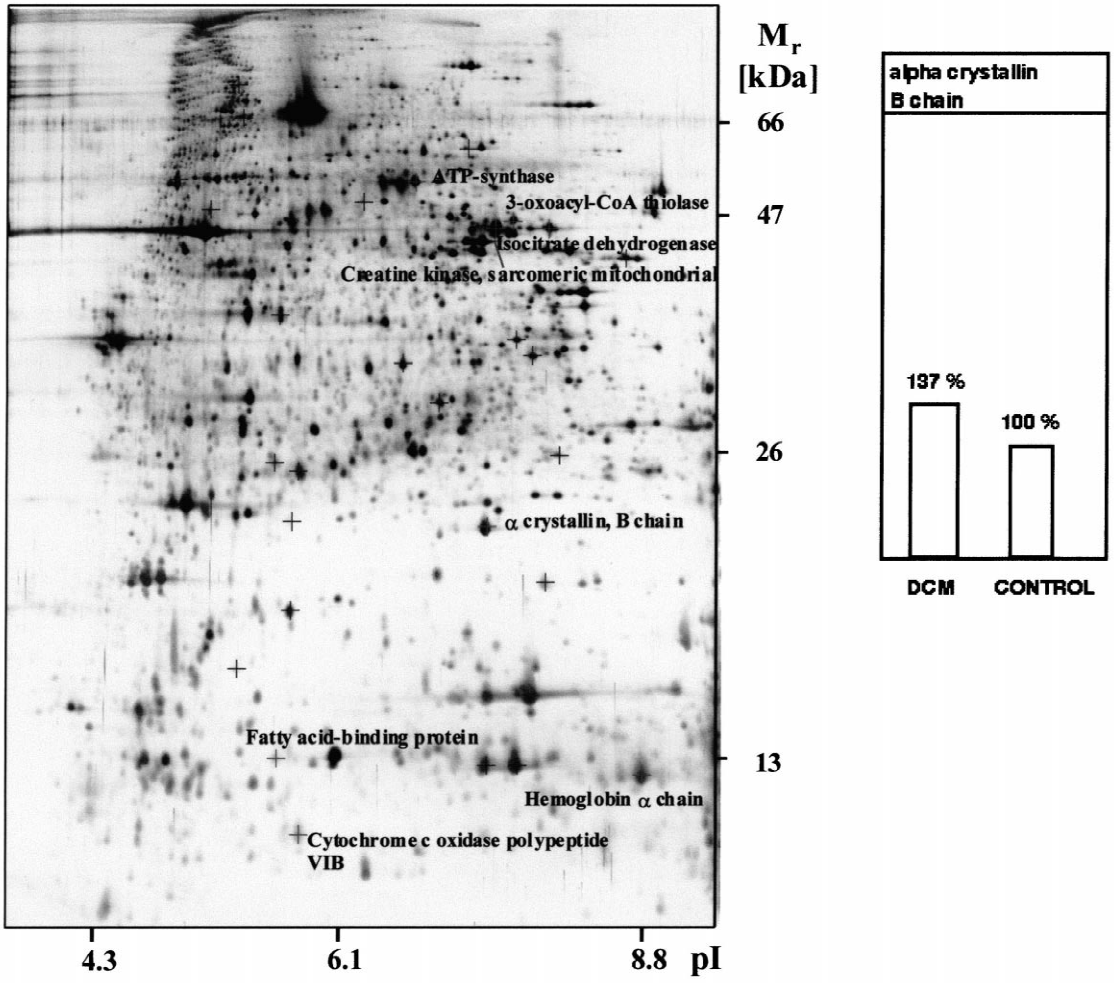

Fig. 1. Standard 2-DE pattern of human right ventricular proteins (HP-2DPAGE; Table 1). Isoelectric focusing with anodic sample application and nonequilibrium running conditions (NEPHGE) was combined with SDS–PAGE in 15% vertical acrylamide gels (23×30 cm×1.5 mm) [4,11]. Proteins were detected by silver staining. Molecular mass and isoelectric point calibration were performed by theoretical values of identified proteins. The given examples of identified proteins (n=70) belong to the group of DCM associated proteins [10–12,26]. Alpha crystallin B chain was found to be increased to 137% in DCM [10].

been used as the support matrix for IEF [19,21,45,82,83], which include a mixture of synthetic carrier ampholytes (CA) in order to establish the pH gradient. Detailed comparison of CA-based patterns generated in separate laboratories may be hampered by discontinuities in the pH gradient resulting from different sources of CA, a drift in the focusing pattern in the late stage of electrophoresis and an instability of the gradient in the higher pH range [84].

An important progress with respect to IEF might have been the introduction of immobilized pH gradient (IPG) strips, where the pH gradient is an integral part of the polyacrylamide gel matrix ([85]; for reviews see Refs. [84,86–88]). Interlaboratory studies of heart, barley and yeast proteins have demonstrated reliable reproducibility of this method [8,87]. The issue of whether to use carrier ampholyte IEF or immobilized pH gradients for the first dimension continues to be subject of considerable discussion [24].

Choices for the second-dimensional separation have not much changed. Either flat-bed (Fig. 2) or vertical systems (Fig. 1) of different size, thickness and percentage acrylamide may be utilized. Vertical settings offer the opportunity to perform multiple slab gel separations simultaneously (up to 20) [84] in the DALT apparatus originally described by Anderson and Anderson [89]. Since the introduction of Ettan-DALT, also individual IPG-strips can be run vertically [87]. Recent developments of second-dimension electrophoresis equipment have even presented ready-made SDS-gels on plastic backing for vertical systems (Amersham-Pharmacia, Uppsala, Sweden).

2.3.2. Complementary protein separation methods

Although there are no actual applications mentioned for cardiac proteomics, the new developments in proteomics technology are so fascinating and promising for the future, that some of them earn a short glance here. Even with the recent advances, 2-DE remains problematic with respect to key issues such as quantification and throughput [24]. For these reasons other separation methods may be warranted. They include capillary IEF and capillary zone electrophoresis [74], ion exchange, size exclusion or reversed-phase high-performance liquid chromatography (RP-HPLC) [7,71,72,90] in conjunction with mass spectrometry to provide a means of direct analysis of protein mixtures [24,28,29]. The combination of continuous free flow electrophoresis (FFE), a powerful liquid-based IEF method, with SDS–PAGE and subsequent peptide fragment sequencing, using on-line RP-HPLC and MS, is capable of fractionating intact protein complexes, and it has the advantage of not being limited by the amount of sample that can be loaded [91].

Affinity chromatography is another extremely selective separation method, exploiting protein affinity for antibodies, specific target proteins or chemical characteristics [7,92,93]. Besides conventional column chromatography or immunoprecipitation, affinity-based separation may be achieved using the newly developed solution and chip arrays in protein profiling. These technologies utilize different capture agents, which can be specific like natural or phage display antibodies [36,37], or unspecific using protein–protein interactions or protein binding ligands [30,32]. Protein microarrays bear the potential for thousands of elements being immobilized e.g. on a chip, with the possibility to retain their ability for selective interactions [32]. A disadvantage of chips covered with antibodies is that only known proteins may be detected, and the discovery aspect of proteomics is fading. Detection and quantification of captured analytes can be managed by fluorescence or biosensor methods, or directly be coupled to MS. Three-dimensional protein arrays in solution (solution arrays) are superior to protein chips, because they do not compromise the functions of binding, and they are more flexible to custom needs. The application of solution and chip arrays in protein profiling has been thoroughly reviewed by Zhou et al. [34].

The problem of protein quantification by MS out of complex samples could be overcome using the newly introduced method of affinity mass tagging reagents [44], or by incorporating surface plasmon resonance (SPR) biosensor systems into MALDI technology [94].

As proteins fractionated by many of these methods retain their native and soluble state, such methods may be directly linked to MS or to in vitro biochemical arrays after separation [7]. But, although very promising, all these new-entries in the field of

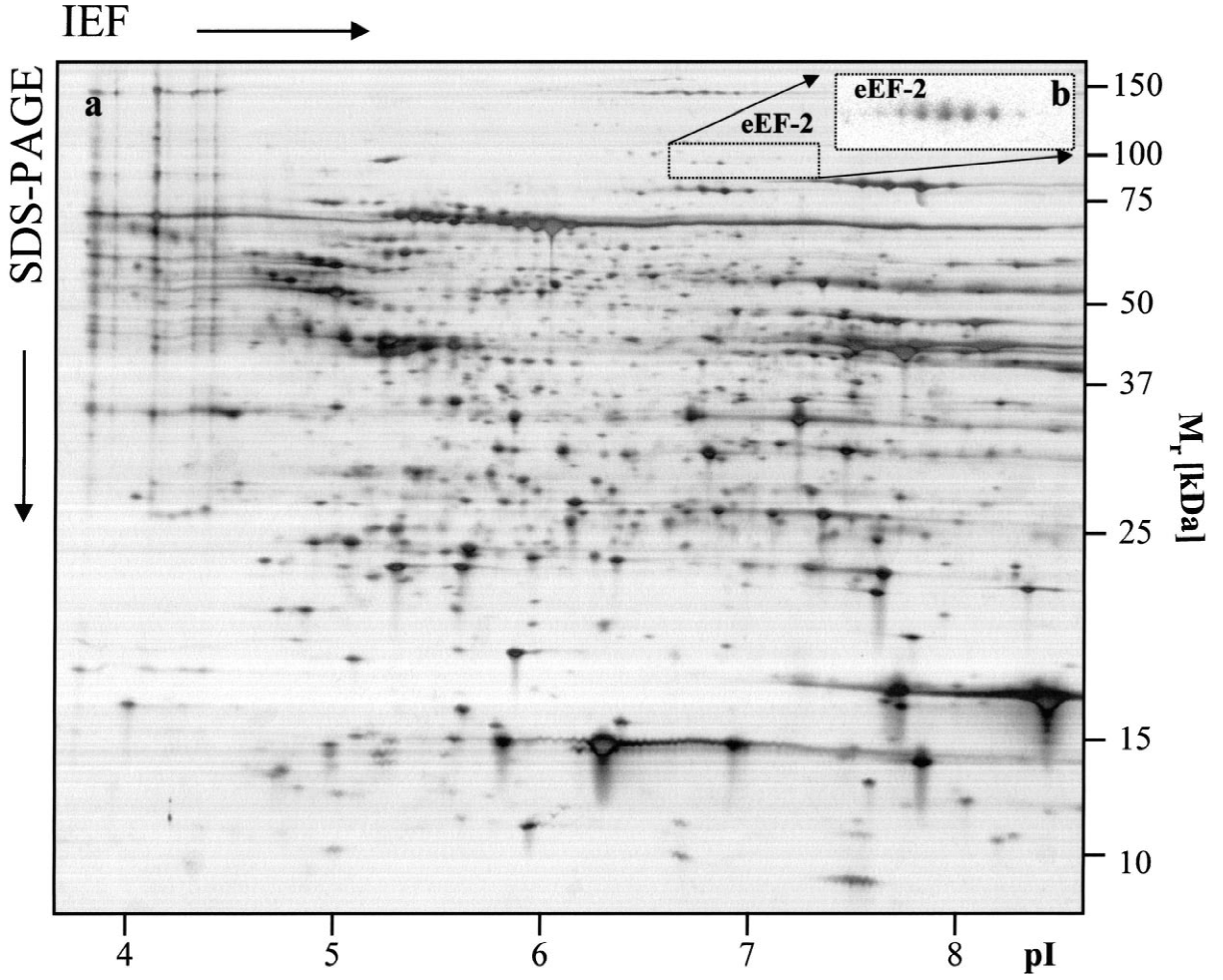

Fig. 2. (a) A two-dimensional electrophoresis (2-DE) separation of 80 μg of human left ventricular extract (control) after [^{32}P]ADP-ribosylation. IEF was performed in a self cast 18 cm IPG-strip pH 4–9 (linear) [106]. The second dimension was run on a ready-made 12% SDS gel (18×24 cm) (Amersham Pharmacia, Uppsala, Sweden) in a flat bed system (Multiphor II; Amersham Pharmacia). Proteins were detected by silver staining; (b) corresponding section of the autoradiogram of an identical 2-DE separation: [^{32}P]ADP-ribosylation specifically labels eucaryotic elongation factor-2 (eEF-2), thereby increasing sensitivity compared to silver staining.

proteomics have yet to equal the 'gold-standard' of 2-DE, both in resolution and in capacity [24].

2.4. Protein detection

After completion of conventional 2-DE, a variety of options for detection of protein spots exist, the more common including Coomassie or silver staining, ^{32}P or ^{35}S radiolabelling or immunodetection [7]. Silver staining with a sensitivity of about 1 ng protein is the most widely applied, but radiolabelling is superior in sensitivity. Especially if a specific radiolabelling method for a protein exists, like Pseudomonas Exotoxin A catalyzed [^{32}P]ADP-ribosylation of elongation factor-2, a key protein of peptide-chain elongation, low-abundant protein species can be visualized on 2-DE of normal protein load, including their variants derived from e.g. posttranslational modifications (Fig. 2) [95].

Coomassie-staining is still very popular—although with a sensitivity about 10–100 times lower than silver and fluorescent stains [96]—because it is

inexpensive, easy to handle and compatible with subsequent protein identification using mass spectrometry (MS) [24]. Colloidal Coomassie Blue G-250 is superior in sensitivity compared to Coomassie Blue R-250.

About 100 different silver staining protocols exist, the majority of published methods to date are based either on silver nitrate (acidic method) or silver diammine (alkaline) for silver impregnation ([24]; for review see Ref. [97]). The latter is not recommended for gels supported on plastic-backings. The introduction of thiosulfate to the developing step has dramatically reduced the background in silver staining and allows thorough development of the image [98]. Another drawback of silver staining is the relative incompatibility with subsequent analytical techniques [96] and the dependence of silver deposition on the protein from the degree of glycosylation [99]. With a modified silver staining protocol, which omits the sensitizing reagents glutaraldehyde and formaldehyde in the silver impregnation step, a commercial silver staining kit (Silver Stain PlusOne; Amersham Pharmacia Biotech) was shown to have some compatibility with in-gel digestion, MALDI and ESI analysis (see below) [100].

Reliable fluorescent stains for protein detection have become available recently and offer an attractive alternative to other detection methods [24]. They have the advantage of superior linearity to protein concentration, a higher dynamic range and equal or higher sensitivity compared to silver stains [8,101]. By binding non-covalently to the SDS molecules coating the protein several SYPRO stains allow reliable quantitative comparison between proteins. The subsequent analysis of the gel by Western blot is possible, leading to the detection of as few as 1000 molecules per cell [8,24]. Several of the newer stains such as SYPRO Ruby are completely compatible with mass spectrometry (MS) [101]. Samples of interest can even be labelled with different fluorescent stains such as cyanine dyes, combined and subject to 2-DE, allowing the detection of protein differences within one gel. This technique is called difference gel electrophoresis (DIGE) [102]. The main drawback of fluorescent stains are the high costs, both for reagents and imaging equipment (UV transilluminator, blue-light transilluminator or laser scanner).

2.5. Image analysis

Adequate image reproduction is essential to properly map detected spots. Image acquisition is most often accomplished with either modified document scanners, charge-coupled device (CCD) camera, laser densitometers and fluorescent or phosphor imagers [8]. For comparative studies identical scanning options have to be maintained for all gels. The resulting images must be converted to a digital format to be applicable to specialized image analysis software [7,8,24]. Some examples for the current generation of system packages are Phoretix 2D, AIDA, Image Master 2D Elite, PDQuest, Melanie III and TOPSPOT. The last one can be downloaded from the internet (http://www.mpiib-berlin.mpg.de/2D-PAGE/) [46].

In general, image analysis entails detection of protein spots, quantification and matching of spots on different gels. Because of the inherent variations in the 2-DE process, a considerable degree of manual editing is still necessary, in addition to the automated procedure of image processing. The degree of confidence placed on the quantitative analysis is highly dependent on the normalization functions of the imaging software, which must compensate variations of sample loading and uptake, staining intensities and distortions during electrophoresis [24,103]. In addition to this, an intensity calibration of the system must be performed, normally using a standardized step wedge, to assure the spot evaluation being within a linear range and to be able to match spots between different gels. Although not absolutely essential, statistical functions are often incorporated into these software packages to deal with the substantial amount of data that is generated by such quantitative analysis of hundreds of matched protein species.

2.6. Protein identification

Some elaborate reviews concerning protein identification in proteomics have been contributed by Jungblut et al. [73], Gevaert et al. [104] and Patterson [105]. Analytical techniques such as immunoblotting, comigration analysis, microsequencing by automated Edman degradation and amino acid compositional analysis [105,106] have all conventionally been used to identify proteins from 2-DE gels.

Protein detection by immunoblotting can only detect known proteins with the prerequisite of highly specific antibodies. In the case of visualization of the antigen/antibody complexes by enhanced chemiluminescence (ECL), repeated stripping and reprobing of one membrane with different antibodies has become feasible. All these techniques were crucial to earlier studies and are still widely used, but are labour-intensive and time-consuming procedures [24]. The combination of high-resolution 2-DE with highly sensitive biological mass spectrometry (MS) and the rapidly growing protein and DNA databases have paved the way for high-throughput proteomics.

2.6.1. Protein electroblotting and Edman sequencing

An automated procedure for chemical N-terminal sequencing of amino acids has been described by Edman and Begg [107]. The modern protein sequencers have sensitivities in the lower pmole range, but, because of N-terminal blockage, only 15–25% of all CBB-stained spots can be identified by this method [11,105]. Chemical procedures for carboxy-terminal sequencing have also been developed, but because of inferior sensitivity this technique is not applicable for protein identification from 2-DE gels [11]. For internal sequencing proteins are digested prior to sequencing, either using an in-gel method or the 'on-membrane' cleavage strategy, proposed by Aebersold et al. [105,108]. The resulting peptides are subsequently eluted, separated by RP-HPLC and individually sequenced (for reviews see [11,104, 105]). The Edman-based approaches are generally slow because every protein spot or peptide peak has to be sequenced individually. In these cases one can hardly speak of high-throughput analysis. However, the first 2-D gel protein databases were generated this way [104].

2.6.2. Mass spectrometric peptide mapping and protein identification

One of the most powerful driving forces of large-scale proteomics was the development of the MALDI-based mass spectrometric identification technology. Comprehensive overviews over this field have been given by Patterson and Aebersold [109], Yates III [110] and Mann et al. [111,112]. In the mass spectrometric approach the characterization of the targeted protein, which is in many cases purified by 2-DE, is achieved via the analysis of a peptide mixture, resulting from an enzymatic or chemical cleavage of the protein of interest (for reviews see [7,11,20,21,24,51,78,110,113]; noncardiovascular see [104,105,109]).

Mass spectrometers consist of three essential parts. The first, an ionization source, converts molecules into gas-phase ions. Once ions are created, they are separated on the basis of their individual mass-to-charge ratio (m/z) by a second device, a mass analyzer, and transferred to the third, an ion detector [110,114]. The most widely used ionization sources are matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) [109,110,115]. The development of these two robust techniques to create ions of large molecules has been essential for proteomics [109,110]. MALDI sources are most frequently coupled to time-of-flight (TOF) mass analyzers, while ESI sources can be linked to analyzers such as quadrupole ion-trap and hybrid quadrupole time-of-flight (Q-TOF) [24,81,114]. Modern ESI-based MS/MS may use LC systems such as capillary zone electrophoresis or very low flow-rate reversed-phase HPLC before ionization to fractionate complex peptide mixtures [7,24,109]. Both methods can determine accurate masses of proteins and peptides in the low to subpicomole range [105] with mass accuracies of up to 0.01% [109].

The first technique generally employed to identify protein species purified by 2-DE is peptide mass fingerprinting (PMF). It is based on the finding that the mass profile of the peptide fragments resulting from a chemical or enzymatic (e.g. using trypsin) cleavage of a protein spot is characteristic of that protein, like a 'fingerprint' [8,24,105]. The set of peptide masses identified using MALDI-MS are compared with possible peptide masses either calculated from theoretical in silico digestion of known proteins or predicted from nucleotide sequence databases [8,24]. The topscoring proteins are retrieved as possible candidate proteins, according to the percentage of matched peptides [104]. The success of PMF is highly dependent on the existence of comprehensive, searchable databases for the species under investigation [24]. This method was successfully applied to proteomics of the heart by several groups, who also established myocardial 2-DE databases [10,11,20,45,46,51]. A breakthrough in this

respect has been obtained by the combination of high-resolution 2-DE gels, enzymatic in-gel cleavage and elution of the peptides with the peptide concentrating device developed by Otto et al. [20].

In the next level of protein identification, called tandem MS/MS, an ionized peptide of interest, selected by the first MS, undergoes collision-induced dissociation (CID), the resulting fragments being analyzed in the second mass etage [7,24]. With MS/MS information on the sequence of peptides is obtained, which represents a powerful tool to discern novel proteins [78,110]. Another main technique for protein identification using MALDI-MS is post-source decay (PSD) analysis [11,18,104]. Here the ion detector is replaced by a so-called 'reflectron field', which reverses the flight path of incoming ions. Fragment ions can so be separated from their intact precursor ions. Although MALDI-PSD spectra are usually complex, sequence tags, i.e. the exact position of one or few consecutive amino acids within the peptide sequence, can in many cases be obtained [18,104]. Yates et al. [116] adapted their SEQUEST algorithm to scan uninterpreted MS/MS or MALDI-PSD spectra against suitably prepared sequence databases, and another computer program was developed called MassTag, which compares theoretical fragmentation patterns with experimental PSD spectra to identify proteins of which PMF could otherwise not lead to protein identification [104,117].

For unknown peptides the above mentioned sequence tag method can be used [18,109] with only 2–3 amino acid residues derived from fragmentation by MS/MS [18]. The sequence tag algorithm is based on the finding that a short piece of partial sequence, together with the molecular mass of the preceding and trailing region of the peptide, is a unique signature of that peptide [18]. Even amino acid substitutions and posttranslational modifications do not prevent the identification of the peptide.

Protein quantification is important in following the changes in gene expression due to disease or a particular cellular intervention [7]. One new technology which allows accurate quantification by MS is stable isotope labelling. This method either grows cultured cells in differently labelled media, or uses labelling after sample preparation, called isotope-code affinity tag peptide labelling [7,44]. Proteins from two different experimental conditions are either reacted with hydrogen or deuterium ion labelled isotope-coded affinity tag reagents. The resulting peptide mass peak heights are indicative of differences in the quantity of that particular protein [44].

Another strategy has been developed leading to direct identification of proteins in complexes. After a proteolytic digestion of a protein mixture and reversed-phase liquid chromatography, the partially fractionated peptide mixtures can be directly introduced into a tandem mass spectrometer [110]. To simplify the peptide mixture applied to LC-tandem mass spectrometry an affinity selection method to capture only cysteinyl peptides was developed [29]. This approach reduces the reliance on SDS–PAGE to separate proteins for analysis. Direct identification of proteins in mixtures has been used to identify components of the yeast ribosome using 2D-liquid chromatography and MS/MS. A total of 80 protein species were identified in a single experiment, at least ten of which were not resolved by 2D-gel electrophoresis [28,110].

3. Proteomics of the heart

The ultimate goal of applying proteomic techniques in cardiovascular research lies in its ability to unravel molecular mechanisms of the disease. In addition to traditional proteomic methods of mapping and identifying proteins, which gave researchers the ability to develop protein databases, the evolution of proteomic tools now facilitates the identification of both the nature and the site of unknown protein modifications. In conjunction with functional data from established biochemical and physiological methods this 'functional proteomics' might further our understanding of the interplay between proteome dynamics and cardiovascular disease.

3.1. Heart 2-DE protein databases

The creation of 2-DE gel protein databases, beginning in the 1990s, has been essential for proteomics of the heart to become a powerful tool in the research of heart diseases. Pioneering proteomic work by the laboratories of M. Dunn and P.R.

Jungblut led to the creation of several online 2-DE databases of human, dog, rat and mouse atrial and ventricular myocardium [1,2,45,47]. The three main databases are HEART-2D-PAGE [2,45,49,118], HSC-2D-PAGE [1,47,79,119,120] and HP-2D-PAGE [46]. In 1995 Kovaljov and coworkers established their own two-dimensional protein database of the human heart with 312 abundant proteins (40 of them identified), but this database was not connected to the World Wide Web and does not meet the rules for federated 2-DE databases [121].

To conform to the rules for federated 2-DE protein databases, such databases have to meet the criteria of accessibility by remote keyword search, linkage to other databases by cross-references and use of main indexes, further accessibility of individual proteins through clickable images and finally, the 2-DE analysis softwares should be able to directly access individual entries into any federated 2-DE database [122]. Over the years many groups have contributed to these databases [20,46,119,120,123,124] (Table 1), but they are still only partially complete with roughly 200 proteins identified. These freely accessible protein inventories are tremendously important, because they provide researchers with a basis for visualization of changes of protein patterns resulting from the conditions of their particular study. Pleissner and coworkers have included a link to heart proteins associated with dilated cardiomyopathy in their database [125], an effort which will hopefully be extended to other heart diseases, introducing 'the next level' of cardiovascular proteomics.

3.2. Proteomics of dilated cardiomyopathy

Dilated cardiomyopathy (DCM) is a cardiac disease, which has been studied most intensively using proteomic tools so far. It is a heterogeneous myocardial disorder characterised by a depressed contractile function and ventricular dilation. In contrast to ischemic heart failure, where the severity usually correlates with the extent of coronary artery lesions, the pathophysiology of DCM is less clear. Genetic factors, myocarditis from infectious agents, autoimmune mechanisms, cytokine activation, toxic damage like alcohol abuse and hormonal or metabolic influences can play a role [126]. Patients with DCM generally have an impaired functional capacity of the heart and—in spite of immense improvements of pharmacotherapy during the last decade—poor long-term outcomes: A mortality of 5–15% per year was reported recently [127]. For medically refractory or progressive heart failure due to DCM cardiac transplantation often remains the only hope.

The precise cellular mechanism leading to myocardial contractile dysfunction in DCM has not been determined, and a direct causal link to a pattern of descriptive findings has yet to be established. Pathophysiological changes already described with biochemical methods other than 2-DE include changes in G-protein mediated signal transduction pathways in human heart failure, e.g. a selective decrease of β_1-adrenoceptor number (for review, see [128]), increased inhibitory G-proteins [129], impaired calcium homeostasis (reviewed in [130]), or

Table 1
2-DE heart protein databases accessible via the World Wide Web

Database	Web address	Source	Protein spots separated	Protein species identified	Last update	References
HSC-2DPAGE	http://www.harefield.nthames.nhs.uk/nhli/protein	Human heart (LV)	~1500	~153	2/98	[1,47,83,**119**,124,139]
		dog heart (LV)	1212	80	10/97	[83,**120**,139]
		rat heart (LV)	1188	30	8/97	[**83**,139]
HEART-2DPAGE	http://www.chemie.fu-berlin.de/user/pleiss/dhzb.html	Human heart (RA, RV)	3300	150	10/96	[2,**9**,45,125] [20,49,118]
HP-2DPAGE	http://www.mdc-berlin.de/~emu/heart/	Human heart (RV)	~3300	70	2/97	[45,**46**]
RAT HEART-2DPAGE	http://gelmatching.inf.fu-berlin.de/~pleiss/2d/	Rat heart	>3000	64	8/98	[123]

abnormalities of contractile proteins with lower calcium-dependent Mg^{2+}-ATPase activity in heart failure [78,130,131]. However the relevance of these findings to the pathogenesis of DCM remains to be determined and many changes may only constitute nonspecific adaptive alterations e.g. due to haemodynamic overload. So far the majority of studies on DCM have concentrated on alterations in specific candidate proteins or metabolic pathways [82]. In this situation proteomics with its main tool 2-DE initially was employed to simultaneously investigate the largest possible range of cardiac proteins in a hypothesis free approach, in order to identify protein alterations specific to DCM.

Because of the multifactorial pathophysiology of DCM a high number of samples separated by 2D-PAGE is expected to be necessary for valid results on relevant proteomic changes of the diseased heart. In the beginning of the 1990s two groups started with a proteomic investigation of DCM [1,2]. The high-performance 2-DE procedure of P.R. Jungblut and coworkers resulted in the resolution of about 3300 myocardial protein species, from which about 150 have been identified so far by amino acid analysis, Edman degradation and MALDI-MS [9,11].

A comparison of right atria from DCM patients ($n=5$) with those from controls ($n=4$) revealed 25 statistically significant quantitative differences, 12 of these protein species having been identified [9,48] (Fig. 1; Table 2).

In another comparative study of samples of right atrial tissue from 13 hearts explanted because of end-stage failure and from 15 control hearts, 52 spots significantly differed in average intensity [125]: Ventricular myosin light chain 2 (MLC2) and heat shock protein Hsp27 have been identified by protein microsequencing and gel map comparison with other databases. In the right atria of the DCM patients the spot intensity of MLC2 was increased to 336% and Hsp27 decreased to 59% compared to the control group (also see Table 2). Therefore these proteins were regarded to be characteristic protein markers for DCM in the right atrium. The upregulation of MLC2 was attributed more to the increase of right atrial wall stress in end-stage heart failure than to altered systolic function of the heart [125]. MLC2 resolves in 2-DE gels as a group of spots in the range of M_r 18 700 and pI 4.7, the intensity of the spots being highly variable in different samples [20]. DCM-associated changes within this spot assembly were confirmed by Corbett et al. [124].

The 2-DE investigation of myocardial samples from the right ventricle revealed that Hsp27 also appears as a huge family of protein species: Using immunostaining 34 [20] or even 59 protein spots reacted with an antibody against human heat shock protein 27 [10,58]. Differences of spot intensity within the Hsp27 group between DCM and controls have been first described by Otto et al. [132]. Knowlton and coworkers detected a two-fold increase of Hsp27 protein concentration in DCM and also an increase in ischemic heart failure (IHF) [133]. A thorough comparison of the Hsp27 protein species pattern of normal ($n=3$), DCM ($n=6$) and IHF human left ventricle specimen ($n=5$) revealed an increase of Hsp27 protein species at a molecular mass of 22–27 kD and a decrease of some Hsp27 spots at 28 kD, possibly resulting from enhanced Hsp27 degradation in heart failure [58]. A down-regulation of Hsp27 actually might be of functional relevance, since it belongs to the group of heat shock proteins acting as molecular chaperones, which are upregulated in a number of cardiac diseases [134]. This again emphasizes that one protein may be represented in several protein species, and therefore variations of a protein sometimes cannot be recognized by evaluation of only one spot [58].

Human myocardium from the right ventricle was subject to 2-DE and resulted in 3239 resolved proteins. In addition to MLC2 and Hsp27, alpha-crystallin β-chain, ATP-synthase [20], β-ketothiolase, isocitrate dehydrogenase and sarcomeric creatine kinase [21] were found to be correlated in intensity with dilated cardiomyopathy (Table 2). Some of these protein species are also represented in the DCM related section of the HEART-2DPAGE protein database, where significant changes of proteins in right atrial tissue samples have been collected from several studies [125].

When chamber-specific composition of human myocardial proteins was addressed, 40 significant quantitative and 5 qualitative differences between right atrium and left ventricle from explanted DCM hearts ($n=40$) were described. Five of the different protein spots were identified as MLC protein species, ATP synthase α chain and phosphoglycerate [135]. This points at the important problem that proteome changes of the right atrium (low-pressure system)

Table 2
Cardiac protein changes associated with human DCM

Protein identified	Kind of change	PTMs	Source	Reference
aconitate hydratase	↓ to 51% (mean)*		left ventricle	[82]
actin	↓ to 11%		left ventricle	[82]
α-actin, cardiac	↓ to 32%		left ventricle	[82]
α-crystallin, B chain	↓ to 64%		left ventricle	[82]
α-crystallin, B chain	↑ to 137%		right atrium/ventricle	[9,20,**48**]
antiheart antibodies (IgG)	↑		left ventricle	[142]
Apolipoprotein A-I	↓to 55%		left ventricle	[82]
ATP synthase, α chain	↑		right ventricle	[9,**20**]
ATP synthase, β chain	↓to 37%		left ventricle	[82]
ATP synthase	↑		right atrium	[9,48]
carbonic anhydrase III	↓to 63%		left ventricle	[82]
creatine kinase M-chain	↓↑ to 74% and 218%**		left ventricle	[82]
creatine kinase, sarcomeric mitochondrial	↑to 232%		right atrium	[9,**48**]
desmin	↓to 35% (mean)		left/right ventricle	[20,**82**,124]
desmin fragment	↓↑ to 59% and 330%		left ventricle	[82]
dihydrofolat reductase	↑to 235%		left ventricle	[82]
dihydrolipoamide dehydrogenase	↓to 36%		left ventricle	[82]
fatty acid binding protein	↓to 61%	acetylation	right ventricle	[9,20,46,**50**]
haptoglobin	↓to 40%		left ventricle	[82]
haptoglobin fragment	↓		right atrium	[9,48]
hemoglobin α-chain	↓		right atrium	[9,48]
hemoglobin β-chain	↓		right atrium	[9,48]
Hsp27	↑		left ventricle	[133]
Hsp27	↓to 59%	phosphorylation	right atrium/ventricle	[20,**125**]
Hsp27, 28 kDa	↓		left ventricle	[58]
Hsp27, 27 kDa	↑		left ventricle	[58]
Hsp60	↓to 47% (mean)		left ventricle	[82]
Hsp60	↑		left ventricle	[133]
Hsp70 constitutive	↓to 42%		left ventricle	[82]
Hsp70 inducible	↓to 38%		left ventricle	[82]
isocytrate dehydrogenase	↑		right atrium/ventricle	[9,48,82]
lactate dehydrogenase, heart	↓to 71%		left ventricle	[82]
malate dehydrogenase	↓to 61%		right atrium	[48]
myosin light chain 1, ventricular	↓to 48%		left ventricle	[82]
myosin regulatory light chain 2, ventricular	↓to 44%		left ventricle	[82]
myosin light chain 2, ventricular	↑to 336%	phosphorylation	right atrium/ventricle	[20,46,124,**125**]
myosin regulatory light chain 2, fragment	↑to 427%		left ventricle	[82]
phenylalanine-4-hydroxylase	↓to 52%		left ventricle	[82]
phosphofructokinase	↓to 58%		left ventricle	[82]
serum albumin, N-terminal fragment	↓to 44%		left ventricle	[82]
transferrin	↓to 35%		left ventricle	[82]
triosephosphat isomerase	↓to 46%		left ventricle	[82]
vimentin	↑to 152%		left ventricle	[82]

*Mean value given, if one protein is represented by several protein species; **indicates that two protein species of one protein show a different kind of reaction in DCM. References in bold give exact values of changes.

cannot be regarded as equivalent to changes of the diseased left ventricle (high-pressure system) [48].

In another comparison of ventricular biopsies from DCM patients ($n=5$) and controls ($n=5$) 258 non-saturated protein spots were found in all 10 gels. Out of these 7 spots were increased 2-fold and 6 spots decreased 2-fold in DCM. Using the Mann–Whitney test, four of these 13 protein spots were identified as

significantly different in intensity in 1994 [50], but they could not be identified at that time [50]. Studies gradually became more sophisticated and the number of cases increased: Left ventricular biopsies were used in a study comparing 28 DCM patients with control donor heart biopsy samples ($n=9$) and 21 cardiac protein patterns from patients with ischemic heart disease (IHD). A total of five spots displayed increased protein amounts in the DCM group compared to IHD heart failure patients ($P<0.01$) and 88 proteins displayed decreased spot intensities [82]. Thirty four of these 93 protein species were identified. Two of the most prominent changes seen were the decreases in MLC2 and in a 50 kDa charge train of spots, previously identified as desmin [47,119]. Ten of 28 (36%) DCM samples showed levels of MLC2 and desmin less than half the mean levels of these proteins in IHD material [82]. A decrease of desmin, which also appears as a spot group lying between the main spots of actin and serum albumin [20], was already described by Corbett et al. [124].

These results are especially interesting, because K.P. Pleissner et al. found MLC2 to be increased in the atria of DCM patients, whereas Dalla-Libera and coworkers also detected lower levels of ventricular MLC2 here [125,136]. Other examples of ventricular proteins decreased in DCM are actin, aconitate hydratase, apolipoprotein A-I and haptoglobin (Table 2). Donor biopsy material displayed similar protein levels to those seen in IHD patients [82].

In the early 1990s troponin T (TnT) was examined in normal compared to failing human left ventricles using Western blot and 2-DE. Two dominant spots of TnT1 and of TnT2 and several minor TnT species were found. The disease-associated increase of the TnT2 isoform was interpreted as an adaptation to abnormal myocardial function [137]. Moreover, some data suggest an enhanced amount of fetal isoforms of troponin T in the failing heart [138].

All the results from studies comparing heart tissue from DCM patients with normal or IHD heart specimen described above are primarily descriptive. But what are the functional implications that can be drawn from different amounts of these protein species? Can these results really give insight into pathophysiological mechanisms of the disease or are they epiphenomena?

Several proteins differing significantly in DCM have metabolic functions like carbonic anhydrase, lactate dehydrogenase or triosephosphate isomerase. Others like alpha-crystallin and heat shock proteins 60 and 70 are implicated in the cell's stress response. Hsp60, also known as chaperonine 60, has also important functions in protein folding and assembly [139]. Phosphofructokinase, which was reduced in DCM, is involved in glycolysis, and the reduction of ATP synthase offers a possible explanation for the low levels of ATP reported in cardiomyopathic hearts [82,140]. Malate dehydrogenase and mitochondrial creatine kinase take part in cardiac substrate utilization and in the creatine phosphate shuttle [48]. Moreover, the decrease in the levels of the regulatory MLC2, which was attributed to an increased expression of a MLC2 specific protease in DCM, was confirmed by the studies of Margossian et al. These investigators were able to correlate the reduction of MLC2 with a twofold decrease in ATP binding and hydrolysis rate in DCM heart myofilaments compared to control samples [141]. The observed reduction in the levels of desmin, which is a major component of sarcomeric Z discs, could contribute to the myofibrillar disorganisation and impaired myocyte contraction in DCM [82,128]. The differential amounts of Hsp27 protein species in normal and failing human hearts may lead to an impairment of actin organization, which is the main physiological function of this protein, besides protecting cells from damage due to several stress factors [10,58].

Moreover, using 2-DE and immunoblotting, reactive antigens were identified, which supports the hypothesis of an immune involvement in DCM. In the sera of 52 patients with DCM a significantly greater frequency of IgG antibodies reacting with MLC1, tropomyosin, actin, Hsp60 and MHC of ventricular myocardium were found than in the sera of patients with IHD [142].

Although the different protein patterns observed in DCM showed no obvious relationship with New York Heart Association (NYHA) functional class or haemodynamic parameters [82], the lower amounts or absence of MLC2 or desmin could have serious consequences for myocyte contractility and therefore cardiac performance. To establish whether these changes lead to or result from a malfunction elsewhere in cardiac cell structure or metabolism, animal

models of volume overload or material from human disease conditions like mitral valve insufficiency might prove informative [82].

3.3. Proteomic results of other human heart or vascular diseases

Reports on proteomic studies concerning cardiac diseases apart from DCM are scarce and are mainly confined to animal models or the use of isolated cardiomyocytes (see below). In an investigation of coronary artery disease (CAD) after heart transplantation those patients who developed early CAD within 2 years of transplantation were compared with those without evidence of CAD at this time [143]. In the sera of patients positive for early CAD, IgM-antibodies had been found reacting with 40 different protein spots on 2-DE separations from endothelial cell extracts. These antiendothelial IgM-antibodies had been correlated with transplant-associated CAD earlier. Using protein microsequencing and immunoblotting 11 of these 40 proteins were assigned to vimentin fragments, tubulin α-1 chain, heat shock protein 60, triose phosphate isomerase and 75 kD glucose-regulated protein (heat shock protein 70 family). It has been concluded that vimentin is one of the target antigens in the development of transplant-associated CAD, possibly mediated by antiendothelial IgM-antibodies of the heart recipients [143]. In another study concerning heart transplantation cardiac antigens were monitored eliciting specific antibody responses in vivo. This may be involved in the process of acute rejection. Some antigens were identified in the sera of heart recipients before transplantation [144]: Tropomyosin, actin, Hsp60, Hsp70 and MHC. Their potential as non-invasive markers for the clinical course after heart transplantation is currently being investigated [6,144].

Human ventricular hypertrophy due to aortic valve disease was investigated by Sutsch et al.: An atrial-like myosin light chain-1 was found to be significantly accumulated in left ventricular biopsies in response to volume or pressure overload [145].

Another interesting approach is the implementation of proteomic analysis to delineate the myocardial intracellular signalling events, that evoke cardioprotection against ischemic injury: Using 2-DE, MS, immunoblotting and affinity pull-down assays, it was found that protein kinase Cε (PKCε), which plays a critical role in ischemic preconditioning, is physically associated with at least 36 known proteins belonging to structural proteins, signalling molecules and stress-responsive proteins [92].

3.4. Animal models of cardiac diseases

An experimental model of heart failure induced by rapid ventricular pacing in dogs resembles human idiopathic dilated cardiomyopathy. Advantages of using this model are the ability to control both the onset and severity of the resulting low output failure. Left ventricular samples of the paced myocardium ($n=5$) revealed 42 decreased and 27 elevated protein spots compared with the control dogs ($n=6$) [146]. Twenty protein species have been identified, 10 of these being associated with mitochondria and energy production (e.g. Hsp60 and Hsp70, creatine kinase M-chain and fatty-acid binding protein decreased) and 2 are cytoskeletal proteins (desmin decreased, desmin fragment increased). Elongation factor Tu was present only in control animals [146]. Interestingly, 7 of these observed changes were equally found in human DCM [82]. Decreased desmin could account for a loss of lateral alignment of the myofibrils and disorganization of mitochondria. A reduction in SERCA2a protein strongly suggests changes in the calcium activation of cardiomyocytes in the paced dogs [146]. When the pI region of these 2D-investigations was increased to the more basic (p*I* 3–10) 23 additional spots could be identified, half of them being mitochondrial or energy production proteins, supporting the notion that the myocardium in congestive heart failure is energy deficient [147]. Creatine kinase M has also been shown as a protein changing in naturally occurring canine DCM [35]. Detailed explanations of the functional implications of the changed proteins in the paced dogs are given [146,147].

Bovine hereditary dilated cardiomyopathy is an autosomal recessive disease, which is endemic in Switzerland and displays important clinical and biochemical similarities to human DCM. Up to now 12 of 35 altered protein species have been identified [144]. The most notable changes were a 5.5 fold decrease of isovaleryl CoA dehydrogenase and a

seven-fold increase of ubiquitin carboxyl terminal hydrolase. The latter enzyme is thought to release free ubiquitin from poly-ubiquitinated proteins, thereby increasing the pool of free ubiquitin in the cytoplasm. Such a large increase of the cellular concentration of ubiquitin could lead to inappropriate ubiquitination of other proteins, targeting them for proteolysis in the 26S proteasome [148]. This finding is in accordance with the suggestion that inappropriate ubiquitination of key regulatory proteins followed by degradation may contribute to the transition of compensated hypertrophy to a decompensated failure state [149]. The same enzyme was found to be upregulated in the heart in human DCM. The specific proteins that become ubiquitinated in bovine DCM have been isolated by affinity chromatography using S5a Sepharose. Of the 26 proteins having been identified so far by MS (e.g. CK M-chain, myoglobin, haemoglobin β-chain), 16 are also markedly decreased in human DCM (M.J. Dunn, Proteomics Forum 2001, Munich).

A 2-DE investigation into cardiac protein levels after chronic ethanol feeding of rats showed significant decreases in the relative amounts of various proteins including several tentatively assigned as Hsp60, Hsp70 and desmin [139] according to the human protein database [1,119]. The relative proportions of actin, vimentin, tropomyosin, MLC1, MLC2 and albumin remained unchanged. It was concluded that the selective effects of chronic alcohol consumption on particular cardiac proteins might explain reduced contractility observed in alcohol-induced heart muscle disease [139]. The comparison of the alcohol effects on the myocardium of normotensive and hypertensive rats showed a general decrease of contractile proteins (predominantly MHC) in both groups, but in the hypertensive rats cardiac CK, malate dehydrogenase and AMP activities were elevated, while values of ATP and RNA were reduced. A greater susceptibility to ischemic damage of the hypertensive rat hearts due to chronic alcohol exposure was concluded from these results [150].

In addition to these studies differences in myosin light chain were observed between fetal and adult rat ventricular tissue, suggesting differences in developmental growth [151].

3.5. Proteomics of cultured cardiac myocytes

Proteomic investigations of isolated cardiac myocytes (CM) are rare and unfortunately a www.accessible database of cultured CM does not exist. The different 2-DE patterns of native and noradrenaline-treated neonatal rat cardiomyocytes were described in 1992, although no protein species could be identified at that time [152,153]. In a cell culture model for cardiac hypertrophy extracts from cultured neonatal rat CM with or without 48 h exposure to phenylephrine (100 μM) were separated by 2-DE. Eleven spots with statistical significant differences were identified using improved procedures for in-gel digestion of silver-stained proteins and MS [52]: MLC1 and MLC2 atrial isoforms were increased, as well as MLC2 ventricular isoform, chaperonine cofactor a, nucleoside diphosphate kinase a and Hsp27. Mitochondrial matrix protein p1 and NADH ubiquinone oxidoreductase 75 kDa subunit were found to be decreased [52]. Increased amounts of MLC2 as well as ventricular presence of the atrial isoform of MLC1, which is normally suppressed after birth, has been associated with cardiac hypertrophy by Northern blot analysis and immunofluorescence as well [52]. Endothelin-induced hypertrophy in neonatal rat cardiomyocytes resulted in a twofold decrease in 21 proteins compared to the levels observed in untreated cells, accompanied by a 30% increase in MLC1 and MLC2 [154].

Some members of the Hsp70 family were found to be exclusively present or increased in isolated human endothelial cells, stressed by heating [155]. It was proposed that these regulatory stress proteins in human endothelial cells might play an important role in ischemic heart disease or autoimmune diseases (one of the reasons of myocarditis and DCM). Damage to endothelial cells, e.g. by hypoxia, cytokines or viral infection is likely to be the initial step in these disorders [155].

Cardiomyocytes from adult rabbits, which had been isolated by collagenase dissociation, were used by the group of J.E. Van Eyk to investigate the effects of preconditioning: Myocardial ischemic preconditioning was recruited pharmacologically using adenosine directly after cell isolation. Proteomic

analysis of cytosolic and myofilament-enriched fractions revealed a novel posttranslational modification to MLC1, consisting of phosphorylation at two sites [59]. The functional implications of this finding and its possible contribution to the preconditioned phenotype remains to be dissected [59].

As the cell culture model permits the detailed examination of cardiac protein composition profiles in response to controlled stimuli, this kind of investigation can be of profound use for proteomics of the heart. The creation of a database of isolated CM from different species and ages would be very helpful in this respect.

3.6. Posttranslational modifications and functional proteomics of human heart diseases

The incorporation of traditional proteome information deduced from mapping and identifying proteins and their posttranslational modifications (PTMs) with functional, biochemical or physiological assays can be called 'functional proteomics' [7]. Especially in acute disease conditions, in which there is often insufficient time to recruit de novo transcription and translation, PTMs would be expected to be the primary mechanism of protein change. But these posttranslational modifications of cellular proteins, that govern physiology of protein interactions and become deranged in disease, cannot be portrayed by gene expression alone. The key technology in this field is mass spectrometry with some recent methodological improvements, allowing the characterization and localisation of many modifications in a single step, although PTMs still provide a major challenge [112,156]. MS is complemented by a number of traditional biochemical techniques (e.g. Edman sequencing, periodic acid/Schiff staining, enzymatic deglycosylation, immunological methods), but they are individually geared towards identifying only one form of PTM [7].

Of several hundreds of known PTMs about 25 are more common, like phosphorylation, acetylation, methylation, palmitoylation, lipoylation, glycosylation or proteolysis [157]. Several web-based software tools are available, which predict PTMs on the basis of mass differences between empirical and theoretical peptide mass fingerprinting data (e.g. FindMod [157], BOLD, NetPhos and GlucoSuite [7]). Phosphorylated proteins, which often indicate activation or deactivation, can be identified by radiolabelling of cells with ^{32}P, enzymatic dephosphorylation, or the use of antibodies specific for phosphorylated protein variants or phosphorylated amino acids [7,158]. Virtually all known cellular signalling pathways are largely mediated through a complex cascade of reversible protein phosphorylation [159].

In the following some examples for the identification of PTMs in cardiac proteomics are presented.

For the identification of MLC1 proteolysis at amino acid residue 19, resulting from severe ischemia/reperfusion, Edman sequencing was successfully applied [160]. In the database of human right ventricular proteins of E.C. Müller and coworkers 1994 a phosphorylation of HSP27 and MLC2 and an acetylation of fatty acid binding protein were indicated, but without notification of any functional implications [46]. Some further examples of the identification of PTMs in cardiovascular diseases are the increase of the proportional volume of two out of three protein spots in pacing-induced canine DCM, which were interpreted as isoforms or possible phosphorylation shifts of these proteins [147]. A reduced phosphorylation of eucaryotic elongation factor-2 (eEF-2), a key protein of peptide-chain elongation, in adult rat ventricular cardiomyocytes (ARVC) in response to 15 ml treatment with insulin was reported. This parallelled an activation of overall protein synthesis [161]. On the other hand an increased phosphorylation of elongation factor-2 together with an inhibition of protein synthesis was demonstrated in ARVC after treatment with isoproterenol by the same group [162]. But these results were achieved with antibodies raised against phosphorylated EF-2 in one-dimensional SDS–PAGE without confirmation using classical proteomic tools so far.

Cardiac Troponin I (cTnT I), an important filament regulatory protein of the myocardium, is extensively post-translationally modified during acute injuries such as myocardial stunning and reversible ischemia/reperfusion. Both a cTnT I degradation pattern and the existence of phosphorylated cTnT I in the serum of patients with acute myocardial infarction was documented [163]. In these studies

myofilament proteins were subfractionated and separated either by SDS–PAGE or HPLC, followed by MS analysis, as an alternative method to 2-DE. Proteolysis products of cTnT I were also found in myocardial biopsies of bypass-patients, which might reflect contractile dysfunction in stunned myocardium [164]. Recently the phosphorylation state of troponin I was reported to be reduced in failing myocardium using a monoclonal antibody and SDS–PAGE [165].

In their study of DCM associated proteins in human right ventricle Thiede et al. [20] were able to identify an acetylation of fatty acid binding protein. This was possible after introduction of a micro HPLC procedure, which separated peptides after tryptic digestion prior to MS. MALDI-MS analysis of the peptide mixture of an acidic spot in the group of MLC2 revealed a phosphorylation of serine 15 in this protein [20]. The differentiation of two dominant Hsp27 spots was again challenging: Whereas the mixture of peptides of both protein species yielded the same MS spectra, a mass difference of 80 in the micro-HPLC diagrams suggested a phosphate group in the more acidic Hsp27 spot [20].

A recent overview over the use of functional proteomics was focused on the signalling module hypothesis in cardioprotection: T.M. Vondriska and coworkers found, that ischemic preconditioning, induced by activation of PKCε, is coupled with dynamic modulation and recruitment of PKCε-associated proteins [78,82].

3.7. Special problems encountered with proteomics of human heart diseases

In analysing human myocardium the inhomogeneity of tissue composition, which includes variable proportions of fibrous tissue, lymphocytes, endocardium, smooth muscle cells and endothelial cells, must be taken into account as possible reasons for quantitative and qualitative changes in protein pattern, that must be differentiated from disease-associated patterns. In addressing these possibilities of sample artifacts Corbett et al. included electrophoretic separations of cultured endothelial and mesothelial cells and cardiac fibroblasts in their study [82]. It was stated that desmin or cardiac myosin were absent in these 2-DE patterns, and the contribution of the small cell population of arterioles to overall desmin levels was regarded to be negligible. On the other hand the intermediate filament protein vimentin, present in all the isolated cells, was detected only in trace amounts in the 2-DE patterns from myocardial protein extracts, so that protein changes of myocardial samples were regarded as disease-specific [82].

Moreover, in the clinical setting there is considerable inter-patient variability, which might hamper the detection of significant differences: Genetic variability, age, sex, metabolic state and medical therapy may result in variations of protein composition [52]. To reduce these effects as much as possible relatively homogeneous groups must be chosen, but, especially in diseases with a multifactorial pathogenesis like DCM, biomedical significance will only be obtained in large-scale investigations including several hundred samples [9]. The availability of these large numbers of samples is however limited [139]. Right atrial and right ventricular myocardium can be obtained easily during a catheterization of the low-pressure system [48], which is performed routinely and at low risk in many cardially affected patients. A left-ventricular biopsy, which is of higher interest for proteomic studies, should not be performed for research interests only, but must be embedded in diagnostic procedures based on clinical demands. Furthermore, the number of biopsies per patient is also limited from medical considerations. As the material of one or two left-ventricular biopsies of 3 to 5 mg tissue each is just sufficient for about 3 large-scale 2-DE gels, the study design must be well structured and the methodology of the laboratory has to be perfectly established.

4. Concluding remarks

Although the elucidation of an organism's genome is the first and most important step towards understanding its biology, and the data created by whole-genome sequencing have significant benefits in many fields outside those of genomics [110], the knowledge of the pure genome will never give full insight into the complex network of functioning proteins, because proteins are the final executors of the various response programs of a cell. 'The genomic

instructions of life are written in the language of nucleic acids, but life is actually *lived* in the language of proteins' [166]. Especially transient information, like posttranslational modifications or interactions among proteins [167], cannot be deduced from DNA sequences alone. Although today we are only on the threshold of proteomics, proteomic studies are poised to enable our conception of proteins and their biological function to transcend to a level that has not been feasible with previous approaches [7,76].

But this does not have to be a contradiction to the paradigms of genetic research: Systematic analysis of the function of genes can take place at the oligonucleotide (functional genomics) or protein level (functional proteomics) [23,111,112,168]. The latter has the advantage of being closest to function.

Especially in the field of cardiovascular science exploitation of the wealth and information generated by genome research holds exciting prospects: With the aid of expressed sequence tag technology and microarray applications [18,23] both known and previously uncharacterized genes, involved in the induction and expression of cardiac hypertrophy and its progression to heart failure, can be analysed simultaneously. A preliminary database for a genome-wide analysis of complex polygenic disorders such as heart failure has been provided [18,22,23].

For cardiovascular research several directions seem to be most promising for the future:

(1) Hypothesis-driven research with careful selection of the specific features of a proteome or subproteome, that provides information about a particular cardiac disease, will complement hypothesis-free approaches. (2) Functional proteomics integrating the time course of protein change, including posttranslational modifications, should allow the reconstruction of the events arising from or leading to cardiovascular changes [7]. Proteomics has already revealed remarkable dynamic changes, that occur at the cellular level, exemplified by the signalling module hypothesis in PKCε mediated cardioprotection [88,92], the phosphorylation of MLCI in pharmacologically preconditioned cardiomyocytes [60], the disease-associated changes in cardiac Troponin T isoforms [137,138,164,165,169] or the impact of cardiomyopathy on the Hsp27 family [58]. (3) The direct analysis of peptides derived from crude protein mixtures [29,168], the analysis of large numbers of even intact proteins by MS, and isotopic labelling of proteins to obtain accurate quantitative data by MS [168] will provide further insights into the intricacies of proteome changes in cardiovascular disease [7] and during cardiac ageing. (4) The study of protein complexes and their spatial organization [39,78], of protein–protein interactions using techniques such as mass spectrometry or the yeast two-hybrid system [42,43,111,167], or the investigation of signalling pathways [170], cellular organelles and compartments [38,40,41,65,71,74,76] will add to our understanding of the pathways of cardiac proteins. (5) As the investigation of human disease entities is complicated by tissue heterogeneity, disease complexity and stage, multimorbidity and age of patients, as well as drug interferences, animal models of human cardiac diseases or age-dependent changes, either transgenic, natural occurring or experimentally designed [52,147,148,150], which mirror e.g. human DCM [52,146–148], will forward our understanding of these disorders [8]. Recent proteomic analysis of neonatal rat cardiomyocytes treated with phenylephrine [52] or endothelin [154] to induce hypertrophic reactions, revealed specific changes of protein amounts.

As this integrated functional genomic/proteomic effort will not be easily amenable for a single laboratory, the feasibility of such studies will most likely require collaboration between laboratories, which are specialized to perform the associated technologies in a reproducible and high-throughput manner [7,24]. A parallel development of high-quality bioinformatic facilities, including the further development of disease- and species-specific databases, will be necessary to complement the armouries of proteomics. It is envisioned that proteomic investigations will eventually lead to novel diagnostic, prognostic or therapeutic markers that can be applied to cardiovascular diseases.

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