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Review

Isoelectric focusing as a tool for the investigation of post-translational processing and chemical modifications of proteins

Elisabetta Gianazza

Istituto di Scienze Farmacologiche, via Balzaretti 9, I-20133 Milan, Italy

Abstract

It has been demonstrated that good agreement may be observed between computed and experimental isoelectric point (p*I*) values when proteins of known sequence are focused under denaturing conditions on immobilized pH gradient IPG slabs, at least in the pH range 4–7.5. Hence, discrepancies between expected and found in this experimental set-up may be reliably ascribed to some kind of post-transcriptional processing, or chemical modification, having taken place in the sample. This evaluation is made easier when the comparison is set between the p*I* of a parent molecule and that (or those) of one to several of its derivatives as resolved in a single experiment (for instance, as a spot row in two-dimensional maps); no previous knowledge is required in these cases about the amino acid composition of the primary structure. The effects on protein surface charge are discussed in this review mainly for two biologically relevant processes, glycosylation and phosphorylation. Then, the p*I* shifts are analysed for some protein modifications that may occur naturally but can also be artefactually elicited, such as NH_2 terminus blocking, deamidation and thiol redox reactions. Finally, carboxymethylation and carbamylation are used to exemplify chemical treatments often applied in connection with electrophoretic techniques and involving charged residues. Procedures to be applied in order to verify whether a given modification has occurred, and often relying on the focusing of a treated specimen, are detailed in each section. Numerical examples on model proteins are also discussed. As an important field of application of the above concepts may be genetic engineering, an exhaustive bibliographic list dealing with p*I* evaluation and structural assessment on recombinant proteins is included.

Contents

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1. Introduction

According to an optimistic conception, proteins synthesized in heterologous 'model' systems (in vitro translation of mRNA, monoclonal antibody secretion by hybridomas, protein overproduction in transfected cells) are expected to be both size and charge homogeneous. A less confident picture still assumes that in experimental settings some steps of the physiological processing may not take place, and the final product should then correspond to simplified 'proforms' as opposed to the mature, complex structures one would purify from natural sources. On the contrary, the proteins produced are often heterogeneous (or microheterogeneous), and in a few instances wrong and unexpected processing does occur.

Worries about the outcome of uncontrolled metabolic pathways have been clearly asserted by National Drug Administrations. Proteins obtained by biotechnological procedures, before they are registered for therapeutic use, need to be shown to be identical with their natural counterparts according to the criteria of both size and surface charge, the latter being assessed by isoelectric focusing [1].

Surprisingly, this point has received only mediocre attention in the scientific literature. In a search for 1984–93 in the EBSCO-MedLine bibliography, 138 entries were extracted by the key words Post-translational 'and' Isoelectric Point, and 62 by Protein Heterogeneity 'and' Biotechnology 'or' Recombinant. In less than 20 out of these 200 items were expected and found properties of a protein compared, and in many instances just the size of the molecule was assessed.

The aim of this paper is to overview the general issue of enzymatic and non-enzymatic modifications of proteins, with reference to their effect on surface charge. Basic concepts and general references will be mostly discussed (in the reference list, *Annual Reviews of Biochemistry and of Cell Biology*, *Advances in Protein Chemistry*, *Methods in Enzymology* and IRL's *Practical Approach* series are most often cited); in keeping with the theme of this issue, specific examples dealing with biotechnological applications are covered in a separate section.

Post-translational modifications may be grouped into two kinds. (a) The first covers changes in a protein primary structure, with proteolysis at peptide bonds [2–4]. It includes the removal of intervening sequences or of leader peptides [5] as for intracellular processing, as well as the action of proteolytic cascades or of 'converting enzymes' in the metabolism of secreted molecules. Most of these events are relevant to biotechnologically engineered proteins [for examples, see [6–10]). Subunits containing unlike peptides usually result in vivo from proteolysis of larger preforms; in vitro, on the other hand, they must be assembled with low yields from individual components. Hydrophobic stretches prompting secretion into the culture medium as much as polyhistidine runs exploited for one-step purification by IMAC are often cloned 5′ to protein coding sequences [11–13]. Formylmethionine is the primary N-terminus of all bacterially synthesized proteins. Although in the latter example, and also in some single amino acid clipping events from the NH_2 [14] or the COOH terminus [15], molecular mass reduction is negligible, these modifications are typically identified by size fractionation techniques, including SDS-PAGE. (b) Attention will be mostly devoted to the second mode of processing, in which the size is slightly affected and no peptide bond is cleaved. This includes both enzymatic [16–18] and non-enzymatic [17,19] covalent modifications in vivo, and in vitro

chemical reactions [20–23]. The list of topics covered is detailed in the Contents. A short bibliographic survey at the end covers the modifications not dealt with in detail.

As a rule, post-translational processing confers upon proteins specific properties that make the holo forms substantially differ from the corresponding apo forms (as discussed in detail for glycoproteins in Ref. [24]). Parameters such as distribution or half-life may be influenced, but most often the very function of the protein is affected. Fine tuning of protein interactions may thus be driven by differential processing along the cell cycle (e.g., [25,26]), at various ontogenetic steps [27–30], in different tissues [31,32] or under varying physiological [33,34] or pathological [35–41] conditions. This evidence, and the possibility of immunological reaction to variant protein forms, explains the concern against improperly processed recombinant proteins for therapeutic use.

With this observation, we are back to isoelectric focusing as an analytical tool to detect protein variants resulting from differential processing. While the discriminating power of the technique will be discussed at length in a later section, it is stressed here that, to be recognized as isoforms derived from a single primary structure, various protein components require to share (and to show) common properties. (a) The simplest parameter is merely quantitative: the only, or the major, components of purified, or partially purified, samples are readily recognized. As trivial as this notation may be, a further common property is to link such a set of peptides, i.e., their purification 'history'. (b) The most complex identifiers, in turn, rely on biospecificity: antigens may be detected by immune reactions, enzymes by zymograms and binding proteins by affinity blotting. In both these extreme cases, 1-D separations are adequate for assessing relationships among protein bands. (c) In general, however, the only similarity one can immediately appreciate rests on similar molecular masses. This corresponds visually to the grouping of protein spots into horizontal, or slanted, rows in 2-D maps. In some cases, the parent molecule and its derivatives are present in similar amounts, in others the apo-protein prevails, and in still other instances one of the modified forms is the most represented. The baricentre of a row may thus correspond to the first (= most acidic, e.g., HSP 27 in HUVEC 2-D [42]), to the middle (= average p*I*, e.g., in serum maps, transferrin or α_1-antitrypsin [43]) or to the last component (= most alkaline, e.g., apoA-I [43]). How to assess the kind of relationships among protein isoforms will be detailed in the main sections of this review. A strategy to screen for coordinate pairs of polypeptides by the computer-assisted analysis of 2-D patterns has been devised by Lemkin et al. [44]. It is based on the assumption that, if there is a post-translational modification in a protein in the transition between two functional states of a biological system, in many cases the sum of the protein concentration of a precursor–product pair in one is equivalent to that in the other. In addition to the identification of candidate pairs with a structural relationship, cues to the nature of the post-translational modification that might relate them could also be derived from comparison of the isoelectric point and apparent molecular mass.

As with all electrophoretic techniques, IEF and 2-D (= IEF + SDS-PAGE), followed by any in situ staining protocol, allow for protein analysis on minute amounts of material, without the need for prior purification. In contrast, 2-D maps may evaluate qualitatively and quantitatively all protein components in a complex sample.

Emphasis is given here to electrophoretic techniques, because with them a wealth of accurate chemico-physical and, in many instances, functional information may be gathered within a short time, with the use of simple and commonplace equipment and of inexpensive reagents. Ready-made IPG slabs make it possible even for inexperienced workers to combine ease with high-quality standards for reproducibility and resolution in p*I* assessment.

NMR may be successfully applied to investigate protein structure [45,46] and even to assess the homogeneity of protein preparations [47]. Very good resolution of macromolecules on the basis of their charge is now being obtained by CE [48]. An application to the identification of

chemical modification sites on metalloproteins can be found in Ref. [49]. Unsurpassed accuracy in mass evaluation is provided by MS [50–52]. Mass variations brought about by the post-translational processing exceed in most instances the confidence limits, as good as 0.001–0.02% with ESI, for a mass range >100 000 [53]. [Δ_{mass} ($100\,000_{NH_2}$) → ($100\,000_{CH_3CONH-}$) = 0.057%; (100 000) → ($100\,000_{PO_4}$) = 0.095%]. Identification of post-translationally modified amino acids is discussed in Ref. [54]; an example of the determination of the glysosylation patterns, disulfide linkages and protein heterogeneities of baculovirus-expressed proteins by MS is given in Ref. [55]. CE and MS more and more often are being coupled off- and on-line in a set-up that duplicates the sequence of 2-D electrophoresis. While it is possible that in the near future these procedures will become standard, and the dedicated equipment will be available to most research institutions, these techniques are just leaving their prototype stage, and their costs still exceed by far the investment required by standard electrophoretic equipment.

Thus, in the meantime, why not exploit available techniques with all of their possibilities?

2. Protein titration curves and p*K* of the side-chain dissociating groups

A titration curve describes the relationship between pH and surface charge of a molecule [56,57]. For a protein, such a curve results from the contribution of the side-chains of different charged amino acids, with various p*K*s and varying relative abundance. When no influence from neighbouring segments of the molecule is exerted on the dissociation of any group, and each charged residue may be treated independently, then the surface charge of a protein results from the sum of individual contributions. This situation is experimentally approached for proteins in their unfolded state when short- and long-range interactions between different parts of the molecule are prevented by high ionic strength and/or chaotropic agents (guanidinium hydrochloride, urea). Under the assumptions above, the course of a protein titration curve may be modelled from its primary structure. The p*K* values for amino acid side-chains and for NH_2 and COOH termini to be used in such a computation are those experimentally derived from a panel of model compounds [57].

Conversely, the experimental assessment of a protein titration curve allows the evaluation of the number and p*K* of its dissociating groups. Two procedures, electrometric [58] and electrophoretic [59–61] titration, may be applied. The former requires several milligrams of a pure protein and a pH meter; the analysis may be carried out over a wide range, including pH extremes. The electrophoretic titration, corresponding to migration of a protein across a pH gradient, is limited to the ca. 4–10 range, where arginine groups are fully protonated throughout. As an advantage, the protein to be analysed needs neither to be homogeneous nor to amount to more than tens of micrograms. What is actually obtained with this technique is a 'pH–mobility' curve. However, for a given protein, the latter is proportional to a true titration, as long as size and shape of the protein are constant across the experimental range, and the viscous parameter dictating u is not affected by pH. In contrast, no direct comparison between the absolute surface charge on different proteins is usually possible, as the above parameter does change from one molecule to another. In fact, this electrophoretic technique has mostly been used to study the pH dependence of binding phenomena (protein–protein [62–64] and protein–ligand interactions, either in solution [65,66] or matrix-bound, as in affinity electrophoresis [67,68]), although inference about structure could be derived by pairwise comparisons between protein isoforms [69,70].

When titration curves are evaluated under native conditions, the discrepancies between computed and found describe the interactions of individual charged groups with their environment, and the connected p*K* shifts. A different approach for the experimental assessment of individual p*K*s into native proteins is through the analysis of the pH dependence of EPR signals

[71]. In a few cases, when the tertiary structure of a protein is known, the pK of specific groups can be computed while the shielding effect of neighbouring residues is taken into account [72–77].

As with any chemico-physical datum, pKs vary with temperature, slightly for acidic and more extensively for basic residues. Reference should then always be made to unambigously specified experimental conditions (see below).

3. Isoelectric point

The pI is a singularity point in a titration curve, corresponding to the pH at which the surface charge of a protein equals zero, hence its electrophoretic mobility is also zero [78–80]. If referred to a random coil, the pI only depends on a protein amino acid composition, and can be computed from analytical or sequence data. In contrast, when interactions are allowed to occur either between amino acid stretches within a protein in its native structure or between ampholytes and appropriate additives in the analysis medium, the experimental pI may be subtly altered. As a rule, when moved to a hydrophobic environment, a group shifts its dissociation so as to favour the uncharged form (i.e., the pK of an acid increases and that of a base decreases) [57]. Whether the result of a structural change (e.g., several well characterized variants of haemoglobin [81,82] or α_1-antitrypsin [83,84]) or brought about by the experimental conditions (D,L-ANS-amino acids and cyclodextrins [85], neutral–neutral protein mutants in urea–detergent [86,87]), these pI changes can be exploited in favourable cases for the resolution of 'neutral' isoforms.

The hypothesis of a random coil for a protein spatial arrangement implies that no restrictions exist on the relative movement between its different segments. This rule would be violated either by the presence of intra-chain covalent bonds, such as with –S–S– bridges, or by structural constraints, such as with turns forced between adjacent residues by proline. Cystein and cystine, both neutral amino acids in acidic to slightly basic buffers, give a differential contribution to a protein surface charge at high pH. A knowledge of the oxidation form of sulfur-containing amino acids is thus required for a reliable assessment of the pI of alkaline proteins.

3.1. Experimental assessment of pI by IEF

Proteins are separated purely on the basis of their pI values by the electrophoretic technique of isoelectric focusing [88], in which amphoteric compounds migrating along a pH gradient stop moving when they reach a region in the separation medium whose pH matches their isoelectric point. Any molecule diffusing out of the isoelectric region becomes charged, to be moved back by electrophoresis to its equilibrium zone, where it concentrates or 'focuses'.

A stable pH gradient within an anticonvective matrix may be established essentially in two ways. The former (CA-IEF) [89,90] makes use of a large number of amphoteric buffers, whose pIs, each slightly different from the next one, evenly fill the pH span to be covered in the experiment. The separation medium, within the boundaries set by the electrodes, or by two conductivity barriers in contact with them (concentrated solutions of an acid and a base, respectively), is buffered at varying pH by the sequence of the buffers (carrier ampholytes, CA) at their pIs. The pH spans covered with this technique vary between 2 and ca. 6 pH units; the gradient is intrinsically unstable with time and may be upset by the presence in the system of non-amphoteric ionic compounds (salts).

Any gradient decay is prevented when the buffering groups dictating the pH are covalently bound within the network of the separation matrix, as with immobilized pH gradients (IPG) [91,92]. These are obtained by the copolymerization of acidic and basic acrylamido derivatives together with acrylamide and bisacrylamide monomers. The gels are allowed to set after pouring a gradient from two limiting solutions whose composition as for the buffering compounds is adjusted to result in a linear pH course between the stated extremes. Gradients prepared in this way may be cast as wide (7.5 pH

units) or as narrow (0.1 pH unit) as required, in order to optimize the resolution of any protein mixture. For specific purposes, non-linear gradients may also be devised and precisely cast [93].

The p*I* of an unknown protein may be experimentally measured after an IEF run by interpolating the focusing position of the relevant band over the actual pH gradient course. For IPGs the latter may be assumed to correspond to the figures computed by modelling, provided that (i) gradient pouring is linear, (ii) the incorporation efficiency is the same for all individual monomers [94], and (iii) the p*K* of the dissociating groups is accurately known under the experimental conditions (effect of temperature [95]; effect of additives [96]). The last point has recently been reassessed by Bjellqvist et al. [97]. They established a relevant pH scale for IPG runs in 8 *M* urea at 20°C, while validating a correspondence between the focusing position on such a gradient and the protein p*I* as computed from its known sequence (see below). Conversely, an experimental evaluation of the pH course in an IPG is hardly possible, e.g., with a surface electrode. Readings may be performed on aliquots of eluted isoelectric buffers when IPGs are run after reswelling in carrier ampholytes (mixed-bed, or hybrid, isoelectric focusing) [98]. The latter approach is standard after CA-IEF, but the accuracy of such measurements is usually low, not only because of the vagaries in the cutting and elution steps but mostly for the systematic effects, which are difficult to compensate for, of differences in temperature (on CA [99,100]; on proteins [101]) and solvent (on CA [99,102,103]; urea on proteins [104,105]) between separation and pH testing.

3.2. Theoretical calculations of pI

At least in the pH range 4–7.5 [106], the accordance between the experimental p*I* of a protein and the figure computed for a random-coiled structure from type and number of dissociating side-chains is so close (within one third to half of a charge unit) as to allow the assignment of a peptide spot in a 2-D map to a known sequence (the second positional parameter, M_r, is also immediately derived from amino acid composition). Any discrepancy from the expected focusing position, and exceeding the experimental error, may then be assumed to imply some structural modification in the species under investigation. By this approach, Bjellqvist and co-workers could identify either four [106] or 18–20 [107] cases of blocked NH_2 terminus; for other proteins, they raised doubts about the peptide being glycosylated, or correctly sequenced [107]. Otherwise, the largest discrepancy was observed for a protein not containing His residues and whose p*I* then depended markedly on the p*K* of the actual N-terminus [107]. Another interesting example of comparison between computed and found p*I* values for mouse MHC class I antigens is discussed in Ref. [108]. As stressed several times, the computation of p*I* is in principle restricted to fully denatured structures. However, for many proteins neither the surface charge distribution nor hydrophilic/hydrophobic balance noticeably influences the intrinsic p*K*s of the charged amino acids, hence the p*I* of the protein and the assumptions above may be extended to functionally folded structures.

For native proteins, 'structural modifications' might imply non-covalent binding as for the holo forms of carrier proteins and prosthetic enzymes, and the covalent (–S–S– bridges) and non-covalent interactions between different subunits within oligomeric structures. For denatured proteins, only covalent modifications are detected after electrophoresis in urea media (except for the 'hydrophobic affinity' effects brought about by addition of detergents to the separation medium; see above).

To be discriminated by IEF, the parent and modified forms of a protein need to differ at pH = p*I* by an integral or fractional number of units in their surface charge. In turn, this charge difference will be proportional to the p*K*–p*I* difference for the charged group involved in the structural change. The extent of p*I* shift for a given charge difference then depends on the buffering power (at p*I*) of the protein, treated as a polyprotic buffer. Less precisely, it depends

inversely on the total surface charge of the protein (at p*I*) and on its size [107].

The discrepancies between theoretical and experimental p*I* values in Bjellqvist et al.'s survey [107] were of the order of a few hundredths of a pH unit, i.e., about one order of magnitude above the current resolving power with narrow-range IPGs (in a side-to-side comparison of different isoforms, a few thousandths of a pH unit are a sufficient ΔpI for unambiguous resolution). These data imply that some of the shifts connected with a covalent modification might not exceed the confidence limit for p*I* estimate; caution is then required for discriminating between the parent molecule and its processed form when dealing with a single protein spot (i.e., when assessing, for instance, whether a COOH terminus is free or blocked).

The finding of peptide spots in a row on a 2-D map (i.e., with similar or identical size, in a slanted or a horizontal row) is diagnostic of the presence of different isoforms deriving from a parent molecule, although relatedness should be positively confirmed by functional (e.g., immunological) or biochemical (e.g., V8 peptide mapping) tests. By the same approach, in order to identify the nature of the bound residue, the number and relative p*I* of the resolved spots are to be compared with number and p*K* of the putative sites for a given post-translational modification. The correspondence of the actual protein pattern with expectation is, however, only a prerequisite to the experimental validation of the hypothesis through chemical and biochemical approaches (specific removal, or analytical identification, of the modifying groups).

4. Post-translational modifications

In surveys on protein processing, tens of amino acid modifications have been listed [16,109]. Many of them are highly specific, whether to a single protein or to a class thereof (just one example in Ref. [110]). Other are commonplace, and among them mainly two processes will be stressed, namely glycosylation and phosphorylation, whose biological role is largely understood. Then some protein modifications that may occur naturally but can also be artefactually elicited will be discussed. Finally, some chemical treatments in common use that are relevant to electrophoretic techniques are analysed.

4.1. Glycoproteins

Sugar moieties are essential in protein targeting to various cellular and extracellular compartments, and take part in signalling and recognition [24,111–115]. Different glycoforms may be tissue-specific [24]. As an extreme case, no Thy-1 molecules are common between rat brain and thymus, despite the amino acid sequence being identical [116]. A marked difference in the glycosylation patterns between two physiological states (baseline vs. acute phase reaction) has been described for a number of serum components (including α_1-acid glycoprotein [117] and α_2-macroglobulin [118]), and also for basolateral membrane protein CE 9 in rat hepatocytes upon administration of the peroxisome proliferation inducer ciprofibrate [119]. Two interesting examples of cytoplasmic non-glycosylated proteins having a secretory glycosylated counterpart are those of ferritin [120] and PAI-1 [121]. Most uncommon, in contrast, is a remodelling in the oligosaccharide chains once the glycoproteins have reached their final compartment; sialic acid removal from serum proteins amounts to a signal for uptake and catabolism [122].

As a rule, the product of protein glycosylation is highly heterogeneous. In animal cells, the last sugar in every saccharide chain is always charged. Sialic acid is not to be found in plants whereas, as a rule, bacteria synthesize no glycoprotein at all [123]. Thus, animal glycoproteins resolve in a 2-D map as a slanted row of spindle-shaped spots. The p*K* of sialic acid is around 3, hence a charge difference between the various isoforms is to be maintained throughout a wide pH range. α_1-Acid glycoprotein is possibly the only example of a protein the resolution of whose isoforms at pH near p*I* depends on the varying extent of sialic acid titration [124]. Removal of sialic acid by neuraminidase treatment

reduces (but seldom abolishes) protein microheterogeneity (some residues are extremely resistant to enzyme digestion). The kinetics of this treatment resolve intermediate bands corresponding to the stepwise removal of sialic acid; their number matches the number of such residues. Sialidase activity is often found in body fluids: careful handling conditions, without repeated cycles of freezing and thawing, are then required for proper sample storage. In order to emphasize the charge shift contributed by amino acid mutation while disregarding any metabolic effect on the glycosylation process and to simplify the electrophoretic pattern, for some glycoproteins, including α_1-acid glycoprotein [125]. genetic analysis is currently performed on the desialylated apoproteins. Complete removal of the sugar chains may be obtained with endoglycosidases (examples in Refs. [126] and [127]). These usually require the protein to be in a completely unfolded state, such as after treatment with SDS followed by saturation with a non-ionic detergent. Changes in M_r always ensue, whereas for sialylated molecules the increase in pI just duplicates what is obtained with neuraminidase alone. For non-sialylated glycoproteins, such as those of vegetable origin, no charge shift is observed [for instance, on 7 S globulin from *Glycine max.* (β-conglycinin); unpublished data].

Enzyme treatment is one of the procedures for assessing the presence of glycyl moieties, to be applied to fully processed molecules. Another experimental approach may be affinity detection after blotting [128] using lectins specific for a given sugar type [129]. Even upon aspecific staining the behaviour of glycoproteins is sometimes peculiar: they may give metachromatic shadows with Coomassie Blue staining or (when heavily sialylated) bind silver very poorly. Sugar moieties are often part of an immunogen epitope, so many neuraminidase- and endoglycosidase-treated proteins lose some or even most of their immunological reactivity. On purified proteins, chemical analysis can qualitatively and quantitatively specify all bound glycyl moieties ([130]; just one example in Ref. [131]).

Synthesis within a controlled experimental system allows one to monitor pI and M_r shifts as the newly assembled protein travels from ER and Golgi to its final compartment [132–135]; to determine the incorporation of tritiated precursors [136,137]; or to prevent glycosylation altogether in the presence of inhibitors [138,139].

Among electrophoretic techniques, CE is becoming a practicable alternative to IEF when 'fingerprinting' oligosaccharides (e.g., [140]).

Non-enzymatic glucosylation will be dealt with later.

4.2. Phosphoproteins

Phosphorylation [141–145] is most often a reversible process, whereupon one (or few) residues are added or released at precise locations, by highly specific enzymes, in response to proper stimuli [146–155]. The holo and apo forms of the affected proteins differ in their biological role, one being the active and the other the inactive form of an enzyme [146], or else one acts as a stimulatory and the other as an inhibitory factor in a regulation process [156]. The in ↔ out flux of phosphate groups thus parallels the on ↔ off switch of a protein function. The current pattern of phosphorylation may thus be highly variable depending on the physiological state of the extracted tissue; it actually defines such a state. Care must be taken to avoid any interference from the extraction conditions, since some phosphate groups are labile; phosphatase inhibitors are available for this purpose [145]. In some cases many sites in a protein may become phosphorylated, but the number of physilogically relevant residues, whose occupancy entails a functional change, is restricted. Addition and removal of phosphate groups might result in an M_r change for proteins resolved by SDS-PAGE [157–160]. On the other hand the one-charge (below pH 6) or two-charge (above pH 7) shift connected with each acidic group always involves a ΔpI, to be resolved by IEF.

The hypothesis that a group of protein bands corresponds to a phosphorylation train may be tested by phosphatase treatment; as a rule, the 'out' step may be performed stoichiometrically

with broad-specificity enzymes [161,162]. In contrast, the 'in' process is usually brought about only by narrow-specificity phosphorylases [163–165]. As a result, whereas reduction of the IEF pattern to a single most basic band is often possible [166,167], the converse, i.e., the 'saturation' of putative phosphorylation sites so as to give a single most acidic protein derivative, is not a biologically sound proposition.

The action of protein kinases, however, may be duplicated in vitro [167]. The experimental set-up in which phosphorylation is tested typically involves the use of ^{32}P [166,168]. Radioactive tagging allows one to appreciate a phenomenon involving low-abundance proteins, such as most of the enzymes and regulatory factors alluded to above. Moreover, only the newly synthesized phosphoproteins enter a qualitative and quantitative evaluation. For the former point, while the physiologically relevant form (or forms) takes up the label, the baseline heterogeneity of the protein is immaterial. For the latter, the incorporated radioactivity is evaluated with reference to a zero background; even when possible, immunological detection or protein staining would require quantification of each species within a sometimes complex train before and after the relevant stimulus, and comparison between the 'baricenter' of the two band rows. Monitoring the 'out' step would be much more difficult with this approach.

Antisera may be developed specific for the phosphorylated versus the apo form of a protein [169]; monoclonal antibodies directed against phosphotyrosine are commercially available. Other experimental approaches to the analysis of phosphoproteins are discussed in Ref. [170]; special interest is devoted to ^{31}P NMR studies [171]. Phosphopeptide analysis is reviewed in Ref. [172].

4.3. Reactions at NH_2 groups

A blocked NH_2 terminus is common finding when Edman degradation is attempted on blotted proteins. A number of residues may react, by chemical equilibrium or enzymatic action, with the free NH_2 group and, while the evidence of a block is easily obtained, no clue is directly available as to the chemical nature of the blocking residue. A stepwise treatment of electroblotted proteins has been proposed in order to expose, whenever possible, the NH_2 terminus [173]. Proteins containing acetylserine or acetylthreonine can be deblocked on-membrane by exposure to TFA vapours and N-formylated proteins by treatment with HCl solution. Pyrrolidone carboxylic acid residues may be removed, with varying efficiency, by pyroglutamate aminopeptidase. N-acetylated proteins are first digested on-membrane with trypsin in order to generate the N-terminal peptide fragment, that is now available to the deblocking action of acylamino acid-releasing enzyme. The percentage of NH_2 terminus-blocked proteins found by different workers in different tissue extracts is highly variable, and artefacts from the sample preparation procedure have been implicated. These might include oxidation, reaction with aldehydes (e.g., HCHO as a contaminant in Tris buffers) and cyclization of glutamic acid to pyroglutamate. Whether a physiologically important modification or an artefact, the blocking is a major nuisance for protein identification through partial sequencing, as it forces the experimenter to resort to protein digestion and peptide purification prior to internal sequencing.

Amino-terminal [174] and side-chain [175] acetylation may be treated together, and also with fatty acylations [176–178], as for their effects on acid–base balance: the removal of an amino group becomes evident only when pI is below pK_{NH_2}. For acidic to neutral proteins, however, the pI shift is easily seen [106]. In contrast to reactions with C_1 or C_2 groups, lipid modifications may be assessed, in newly synthesized proteins, by monitoring the incorporation of ^3H-labelled fatty acids (myristate [179], palmitate [180,181]).

Core histones can be reversibly acetylated at distinct lysine residues within the N-terminal protein domains [182]; this process is assumed to be involved in changes in chromatin structure and function during different nuclear processes. A dynamic equilibrium between apo and modified forms is maintained by two enzyme ac-

tivities, histone acetyltransferase and histone deacetylase [183]. Owing to their high p*I*, the histones cannot be run to equilibrium in CA-IEF and bind to the matrix on IPGs [184] (Righetti et al., however, subsequently presented data about the feasibility of alkaline IPG not interfering with histone migration). Their acetylated or otherwise modified derivatives are then resolved either by acid–urea–Triton electrophoresis [185] or by CE [186].

A peculiar example of reaction with the NH_2 terminus, and of a diagnostic use of IEF, involves haemoglobin and glucose. The adduct formed rearranges to give a chemically stable product, which makes the overall process irreversible [187]. The equilibrium between free and bound glucose depends only on its concentration: the determination of the glucosylated haemoglobin thus evaluates the average glycaemia over the lifespan of the protein, which amounts to several weeks. This makes glucosylated haemoglobin (HbA_{1c}) a valuable index in the follow-up of diabetic patients [188,189]. However, an important physiological property of the reacted protein, namely the O_2 dissociation curve, is also modified. In diabetes, the effects of generalized protein glucosylation are manifold [190]. One of the most serious involves the alteration of the filtering properties towards either anionic or cationic molecules by the kidneys, which behave in fact more as an ion exchanger than as a sieve. By detailing this example and that of histones above, it is intended to stress that the issue of p*I* shift after chemical modification treated so far only from a chemico-physical standpoint may also have major physio-pathological impact. For proteins other than Hb, non-enzymatic protein glucosylation [191–193] involves lysyl residues, instead of NH_2 termini (albumin [194], histones [195]).

4.4. Reactions at COOH groups

Deamidation is a most general phenomenon connected with protein ageing: for molecules with long half-lives, its quantification involves evaluating the protein turnover [16]. Δ (charge) for the transition $CONH_2 \rightarrow COOH$ varies across the pH range 3.5–5.5, corresponding to the titration of the carboxyl group. As a result, the p*I*s of parent and modified molecules may be difficult, or impossible, to sort out for very acidic molecules. Moreover, the electrophoretic migration of the two forms, differing at high pH, become more and more similar at low pH: at pK_{COOH}, the distance between the two protein bands is reduced to about half in comparison with $pH = pK + 1$, and becomes negligible at $pH = pK - 1$. This observation may in principle be exploited to verify whether two protein isoforms actually differ by a deamidation step: the electrophoretic titration [57–59] of the proteins to be compared is run across an acidic pH range, and the shape of the two curves is analysed. The same feature would be observed also for neutral → acidic amino acid mutations. As a rule, however, the deamidated side-product is a minor percentage in comparison with the amidated parent molecule, while two allelic variants in a protein polymorphism are expressed with the same abundance. Another indirect test for deamidation of Gln and Asn residues may rely on the differential specificity of V8 protease for Glu-C and Asp-C in different buffers, in comparison with the intensitivity to digestion of the amidated counterparts [196,197]. Another analytical approach, were both isoforms purified in substantial amounts, might involve the reaction with [^{14}C]glycine methyl ester in presence of soluble carbodiimide (resulting in the amidation of free COOH groups) [198]. Extensive chemical deamidation at alkaline pH, or blocking of free COOH, would result either in two proteins with lower but identical p*I*, or in polycations with identical mobility. These reactions could be performed on the unresolved isoforms, but neither of them is quantitative so as to permit an unequivocal interpretation of the results. Sequencing of the relevant peptide is then a complex yet reliable approach to assess the nature of the structural difference between the proteins under investigation. Extraction and treatment under alkaline conditions might result in artefactual protein deamidation; low temperature and as short as possible exposure times are thus essential to minimize deterioration.

The occurrence, detection and biosynthesis of carboxy-terminal amides and isoprenylation and

methylation at carboxy-terminal cysteine residues are discussed in Refs. [199] and [200], respectively, and exemplified in Ref. [201].

4.5. Reactions at SH groups

Many heteropolymeric proteins with interchain –S–S– bridges derive from continuous sequences processed to their quaternary structure through peptide bond hydrolysis, with or without the removal of an amino acid stretch [2–4]. In fewer cases, independently synthesized subunits are covalently linked to one another after their assembly [202]. In a single-chain protein, the position of the disulfide bridges is dictated by the folding process, which in turn depends on the primary sequence; in vitro, air oxidation is adequate to form disulfides between the appropriate Cys pairs [203,204]. Under physiological conditions this step, which requires hours when testing a fully denatured and reduced protein, is completed alongside the translational process [205,206]. Cycling between reduced and oxidized (thiol SH $\rightleftharpoons$ disulfide –S–S–) forms is thought to take part in metabolic regulation through the redox control of enzyme activities [207]. However, heterogeneity such as for the oxidation state of a purified protein is often the result of an artefact, most likely oxidation by atmospheric oxygen at alkaline pH. For a purified protein, if a single SH residue is available per molecule, then only dimers may be formed, whereas a more complex situation might result in multiple banding [208]. While the heteropolymers are expected to have p*I*s intermediate between those of the parent components, the homodimers are indistinguishable from the monomeric form as long as the dissociation of SH does not contribute to the protein surface charge. Thus, acidic and neutral proteins during an isoelectric focusing separation are not exposed to such a pH as to elicit an artefactual oxidation, nor can this process, had it taken place beforehand, be detected by electrophoresis [209], although a functional test, such as zymogramming an enzyme preparation, may reveal a reduced specific activity. In contrast, for alkaline proteins, both the conditions of migration favour the oxidation process and the resulting p*I* shift can be identified by IEF [210,211]. While buffer addition with low concentrations of a reducing agent, for instance DTT, is compatible with zonal electrophoresis, their weak acid nature makes thiolic reagents hardly compatible with isoelectric focusing. In fact, when incorporated into an IPG gel, the additive tends to migrate at $pH < pK$, and hence it does not contribute to the reducing potential, whereas the non-depleted portion interferes with buffering power and pH course in the alkaline part of the gradient [209,212]. Most recent prescriptions to avoid protein oxidation on IEF include reduction of the IPG matrix with ascorbic acid [213], running under paraffin oil to exclude access of O_2 [210,214], and a certain migration of thiolic reagent from the cathodic strip during the last phase of the run [214]. With CA-IEF, even the low concentrations of thiolic reagents contributed by sample buffers distort the pH gradient above $pH \approx 7.5$, with loss of alkaline proteins, when applied, as customary, near the cathode [209,212].

One alternative to the above problems could be to dispose altogether of reducing agents both in the sample and in the gel by alkylating any free SH on proteins. This approach has been proposed for SDS-PAGE samples in order to avoid Cys reoxidation and erratic banding patterns [215]. Moreover, treatment with iodoacetamide has been suggested as the final equilibration step between the first and second dimensions of a 2-D separation, in order to reduce background staining with many silvering procedures [216]. Carboxymethylation for IEF, or 2-D mapping, has been discussed a few times, but no consensus protocol could be arrived at. When applied to complex mixtures of proteins, such as whole tissue extracts, reduction and alkylation with iodoacetamide result for most of the components in neat, single spots. However, in comparison with a run of standard sample preparations, some spots disappear, or change their focusing position, or grossly increase their staining intensity. No simple trend can be observed for these anomalies, and none of the possible explanations for these findings (incomplete selectivity of the reaction towards SH versus NH_2 groups; partial deamidation of the

akylating reagent; differential silver deposition on the alternative nucleophilic centres, SH and –S–C–) is easily proved for any specific case [209]. These perplexities notwithstanding, carboxymethylation has been proposed as an analytical tool. Treatment with different ratios of iodoacetamide and iodoacetic acid results in the formation of a train of bands differing by one charge unit, and whose number exceeds by one that of Cys residues [23,217]. Intrinsically restricted in its application to charge-homogeneous proteins, and largely outdated by current sequencing techniques, this suggestion still deserves mention because, as is usually the case with techniques relying on electrophoretic separations, it may be applied even to incompletely purified proteins, as long as the relevant bands may be unambiguously identified, while allowing one to count integral numbers of amino acid residues per protein molecule, in contrast to standard analytical protocols following HCl hydrolysis. The above procedure introduces the concept of chemically derived charge trains, that is most often realized by stepwise carbamylation. SH and –S–C– forms of a protein migrate to the same position in IEF when their p*I* is acidic to slightly basic, but differ in their surface charge at alkaline pH. Monomeric and polymeric forms of a protein migrate with different mobility in a sieving medium under native conditions: only the fastest migrating band will be detected after addition of just SDS if the inter-subunit interactions are non-covalent in nature, whereas a reducing agent will be required if there exist cystine bonds. For a peptide with intrachain –S–S– bridges, the apparent M_r in SDS-PAGE is lower than expected from sequence data in the absence of a reducing agent. After reduction with β-mercaptoethanol, the estimated value fits the logarithmic relationship with R_F, and seems to grow larger upon carboxymethylation.

4.6. Carbamylation

The carbamylation protocol involves boiling homogeneous proteins, of neutral to alkaline p*I*, in a concentrated urea solution for increasing lengths of time [218]. From the decomposition of urea, carbamic acid is formed, which reacts with free NH_2. A series of protein derivatives of decreasing p*I* are obtained, evenly spaced along the p*I* axis at first, then focusing closer and closer as the influence of the residual buffering groups (His and Glu/Asp) becomes more important at pH = p*I*. Individual spots are conventionally labelled as 0 (parent molecule), $-1, \ldots, -n$ (loss of 1 to n positive charges). These mixtures of modified proteins have been used for years as landmarks for calibrating the pH axis in 2-D maps [218,219]. Although extremely efficient [220], the classical protocols for 2-Ds with CA-IEF in capillary tubes could not achieve absolute positional reproducibility in the spot pattern, for a number of reasons, e.g., deformability of the IEF gel rod, pH drift with time and gradient distortion from salts or thiolic reagents in the sample buffer. At the same time, for the natural protein spots that could be taken as landmarks, being common to many if not all the tissue extracts, the p*I* in urea was not known with accuracy [218]. The inclusion of the carbamylated proteins was to set a reference grid, not intended to specify a chemico-physical parameter such as p*I* but merely a relative position. Virtually all of these problems have now been solved with IPG technology. A relevant pH scale has been detailed [97], and accurate pH courses for many formulations covering narrow to wide pH gradients have been computed and published [93,221–223]. Moreover, the reproducibility of gradient pouring has been tested, and the positional reproducibility of a 2-D map, when IPGs supported on GelBond plastic backing are used in the first dimension, has been found to be better than 0.5 mm [224]. This allows one to read directly the p*I* of a protein spot, and its M_r, from its absolute position in the x–y plane. Moreover, the recent work by Bjellqvist et al. [107] established the correct p*I*, under denaturing conditions, for a number of ubiquitous proteins. In another context, carbamylation by treatment with sodium cyanate had been proposed for reducing the average p*I* of IgGs to be used in electroimmunodiffusion (Laurell's rockets) for the determination of alkaline proteins, whose electrophoretic mobility would be very

low at pH 8.2 (i.e., at the average p*I* of native IgGs) according to standard protocols [225].

Maleylation and its effects on p*I* and SDS-PAGE mobility have been discussed in Ref. [226].

4.7. Miscellaneous

In the bibliographic search mentioned in the Introduction, although most references dealt with glyco- and phosphoproteins, in three cases the authors dismissed any covalent modification to the protein under investigation and in 17 they had evidence of a post-translational processing but could not specify its nature.

An amide-linked ethanolamine–phosphoglycerol is reported in Ref. [227]; an example of methylation [228,229] is discussed in Ref. [230]. ADP-ribosylation [231,232] and ubiquitination [233,234] were analysed in histones ([235] and [236], respectively). Of the possible effects of covalent coenzyme binding to proteins [237,238], only one old example could be found [239].

5. Numerical examples

Although this review focuses on theory, while urging to experiment, it was felt that at least one figure with a few numerical examples were needed. Three proteins were considered as model structures, A, B and C (Fig. 1), differing in their amino acid composition (top panels, and left to right) and buffering power at p*I* (see below). For each of them, Δp*I* was computed [240] for a number of hypotethical post-translational modifications. Rounded figures were used for all p*K* values (see upper right panel; standard one-letter abbreviations are used for amino acids, COOH and NH_2 represent protein termini and H_2PO_4 and HPO_4^- indicate pK_1 and pK_2 of a phosphate group). In order to reduce the number of variables to be taken into account in the calculation [240], D and E counts were combined, and no Y was included in the hypothetical sequences. The postulated modifications are marked in Fig. 1 as follows: (1) for glycoproteins, presence of 1 sialic acid; (2) for glycoproteins, presence of 3 sialic acids; (3) for glycoproteins, presence of 5 sialic acids; (4) for phosphoproteins, presence of 1 phosphate group; (5) blocked NH_2 terminus; (6) deamidation of 1 Q/N group; (7) monomer with thiol redox as –S–S– plus SH; (8) dimer assembled via 3 –S–S– bridges; (9) for carbamylated proteins, reaction at 1 K; (10) for carbamylated proteins, reaction at 2 K; (11) for carbamylated proteins, reaction at 4 K; (12) for carbamylated proteins, reaction at 6 K.

The associated Δp*I* are plotted clockwise for each model protein (lower panels, from left to right); since these values vary by over three orders of magnitude, a logarithmic scale had to be used for their representation (bottom right).

In protein B, p*I* = 5.165, $\beta_{pI} = 5.68$ mequiv l^{-1} pH^{-1}, at 1 m*M* concentration, the p*I* shifts observed for glycosylation, phosphorylation, NH_2 blocking and carbamylation differ from one another by a maximum of 0.006 pH unit, since they actually amount to the addition, or to the removal, of one unit charge, either negative or positive. Owing to the buffering effect of D and E at and below p*I*, in stepwise processes the shifts become lower and lower, to approach a constant value. One hypothetical deamidation, in contrast, results in a fractional surface charge variation, hence the substantially lower p*I* decrement for the $CONH_2 \rightarrow COOH$ transition. No influence is observed from the redox state of C residues, since pK_{SH} is much higher than p*I*.

The same trend is observed for protein A, p*I* = 4.500, $\beta_{pI} = 14.3$ mequiv l^{-1} pH^{-1}, except that the Δp*I*s are much lower both in absolute terms and as differences among various one-unit changes. Moreover, the p*I* shift associated with a fractional charge variation (i.e., for the deamidated protein) amounts to a much lower percentage than in the instance above. This is, of course, the effect of the higher buffering power at p*I* allowed by the much larger number of D and E residues in this structure.

Conversely, the buffering power of protein C is very low, $\beta_{pI} = 5.02$ mequiv l^{-1} pH^{-1}, at and below the isoelectric point, p*I* = 9.014, and the shifts between subsequent steps in glycosylation and carbamylation become larger and larger. At

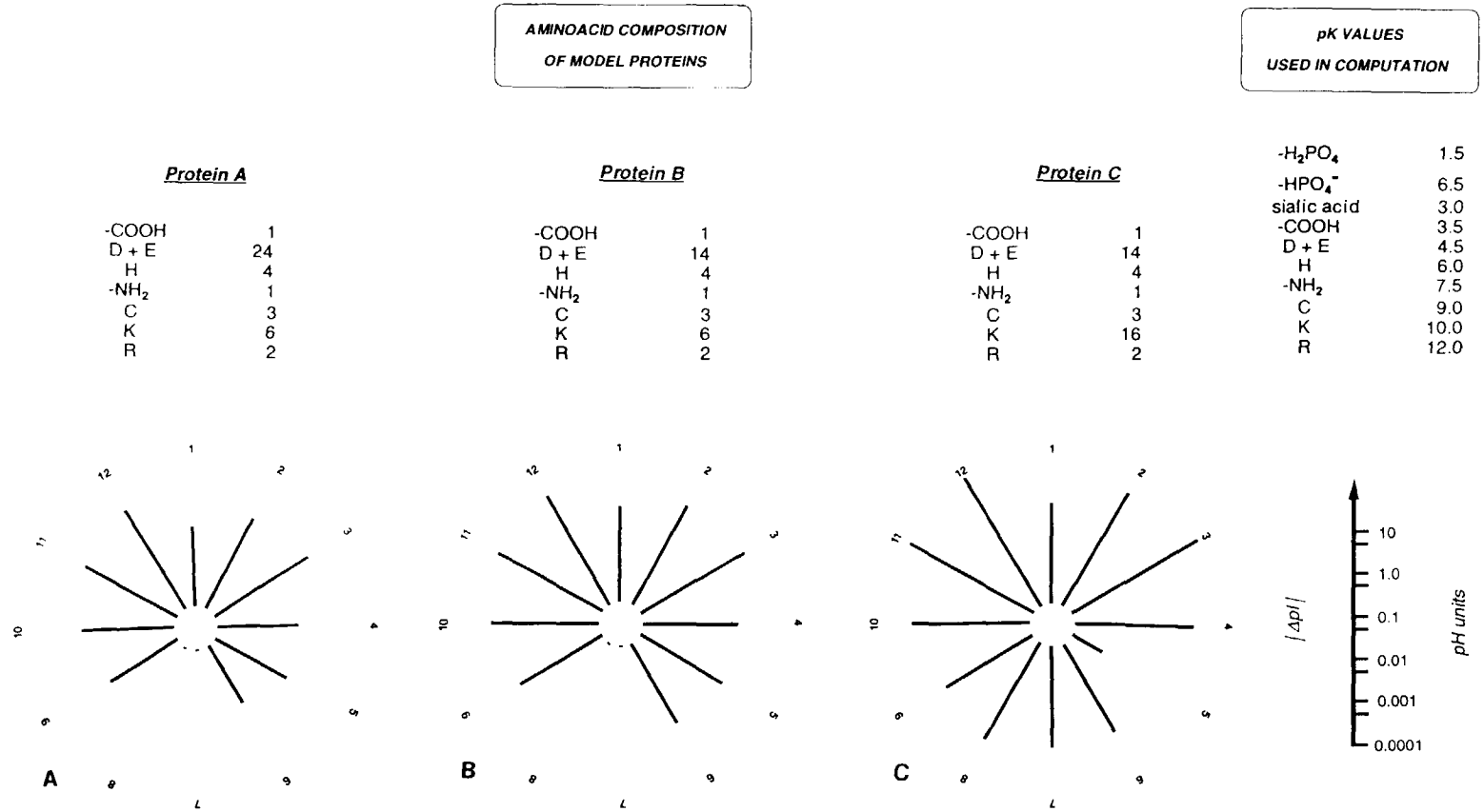

Fig. 1. Expected ΔpI for various post-translational modifications in different hypothetical protein structures. The isoelectric point for the three model proteins A, B and C, whose amino acid compositions are given in the top panels, was computed by the program described in Ref. [233]. The pK values used in the calculations are tabulated on the right. New pIs were evaluated after the following changes, corresponding to the simulation of some kind of post-translational modification: case (1) addition of 1, case (2) 3 or case (3) 5 sialic acid residues; case (4) addition of 1 phosphate group; case (5) removal by blocking of NH_2 terminus; case (6) deamidation, i.e., addition of 1 D/E residue; case (7) removal by oxidation of 2 C residues; case (8) removal by oxidation of all C residues resulting in protein dimerization; case (9) removal by carbamylation of 1, case (10) 2, case (11) 4 or, case (12) 6 K residues. The resulting ΔpIs are represented clockwise according to a logarithmic scale (bottom right) in the diagrams of the lower row. See text for more details.

pH 9, K is not 100% protonated and carbamylation thus involves fractional charge decrements. NH_2 terminus blocking results in a pI shift of only 0.006 pH unit, as pI is much higher than pK_{NH_2}. Phosphorylation brings about a two unit charge variation, whereas the removal (by oxidation) of each SH group corresponds to one-half charge difference (pK_{SH} equals pI).

6. Recombinant proteins

The reports in Table 1 [241–260] are the only ones from the bibliographic search where the issue of charge heterogeneity of recombinant proteins was addressed. In most cases, however, the properties of the biotechnological product were just assessed instead of being compared with the natural product. The chemico-physical parameters of the latter were possibly hardly known in some instances, and gene cloning for heterologous expression was actually a short-cut in comparison with a lengthy and cumbersome purification strategy.

Most of the problems connected with gene expression technology, including co- and post-translational processing, modification and secretion in different heterologous systems, are dis-

Table 1
Comparison between natural and recombinant proteins

Protein	Gene source	Expression system	Correct properties	No glycosylation	Heterogeneous glycosylation	Incorrect properties	Ref.
Myosin light chain	*Drosophila*	In vitro	×				[241]
Acid phosphatase	Man	In vitro	×				[242]
Peptidyl prolyl isomerase	Man	*E. coli*	×				[243]
M protein	Parainfluenza virus	*E. coli*				×	[244]
α-Interferon	Man	*E. coli*		×			[245]
Structural proteins	Semliki Forest virus	Yeast				×	[246]
G protein βγ-subunits	Man	Insect cells	×				[247]
Interferon γ-receptor	Mouse	Insect cells			×		[248]
Manganese peroxidase	*Phanerochaete chrysosporium*	Insect cells				×	[249]
HLA-B27 + E3/19K	Man adenovirus	Insect cells			×	(×)	[250]
Interleukin-9	Man	T-cell lines			×		[251]
T1	Mouse	Fibroblasts			×		[252]
gp130	Man	Mouse melanoma	×				[253]
Fib2 of fibronectin	Man	Mouse L cells			×		[254]
TGF-βIII receptor	Rat	COS cells			×		[255]
Tissue factor	Man	CHO cells			×		[256]
Proenkephalin	Rat	CHO cells			×	(×)	[257]

cussed in Refs. [258] and [259]. From the list, the number of cases for which correct properties are observed equals the entries for which incorrect properties are reported. It is no surprise to find that no glycosylation occurs in *Escherichia coli* [243]. Complete fidelity to the model, as might be expected, is obtained when a human protein is expressed by the same histotype in a different animal species [253]. The glycosylation pattern of virus proteins is known to be host-specific [24]. However, especially interesting is the observation that when expressed in *Saccharomyces cerevisiae* the E1 envelope protein of Semliki forest virus receives yeast-characteristic outer-chain glycans [246]. Phosphorylation is reported in Ref. [257].

Two examples of fusion, or chimeric, proteins found heterogeneous with respect to p*I* and/or to M_r are discussed in Refs. [259] and [260].

Finally, the case of a shift in isoelectric point between cellular and secreted forms of a monoclonal antibody against gp-41 of HIV-1 virus is reported in Ref. [261].

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Abbreviations

CA = carrier ampholyte; CE = capillary electrophoresis; 1- and 2-D = one- and two-dimensional electrophoresis; EPR = electron spin resonance; ER = endoplasmic reticulum; ESI = electrospay ionization; HUVEC = human umbilical cord endothelial cells; IEF = isoelectric point; IMAC = immobilized metal chelate chromatography; IPG = immobilized pH gradient; MHC = major histocompatibility complex; MS = mass spectrometry; NMR = nuclear magnetic resonance; PAI-2 = plasminogen activator inhibitor-2; p*I* = isoelectric point; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; TFA = trifluoroacetic acid; Thy-1 = cell-surface glycoprotein, member of the immunoglobulin superfamily; u = electrophoretic mobility.

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