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INTERNAL CHARGE STANDARDIZATION FOR TWO-DIMENSIONAL ELECTROPHORESIS

B. J. Hickman, N. L. Anderson, K. E. Willard, N. G. Anderson

Molecular Anatomy Program, Division of Biological and Medical Research,
Argonne National Laboratory
Argonne, Illinois 60439, USA

Introduction

Two-dimensional electrophoresis employing isoelectric focusing in urea in the first dimension and electrophoresis in the presence of sodium dodecyl sulfate in the second (1-4) gives a high degree resolution of the proteins in a sample. However, it is necessary to be able to specify the position of each spot with precision. Ultimately, spots should be located to the limits of resolution of the system to establish positional identity of spots on two different gels. If spot locations were to be compared by simple measurement, spot positions would have to be reproducible on consecutive gels within a fraction of a millimeter. This is not at present feasible with current systems and equipment. Alternatively, pH measurements may be made along the isoelectric focusing gel and molecular mass standards may be run along the edge of the second dimension SDS gel, and pH and molecular mass plotted along the edges of the pattern. This procedure is frequently used but provides only an approximation of the correct values and the methods are tedious and inconvenient for our purposes. Measurements of pH in 8 M urea are not fully understood, and cannot be made at a large number of points and related with precision to the final position of each spot, and hence do not show the small and short-range nonlinearity of ampholyte-generated pH gradients. Molecular mass indications based on standards run along the edge do not take into account any curvature of the pattern, and almost always employ a very limited number of proteins. We have solved the problem of molecular mass standardization by using heart muscle proteins added to the agarose used to seal the first dimension gel in place. The result is a large number

of horizontal lines (over 70) ranging over the entire pattern which have been calibrated for general use (5, 6). The problem of internal charge (or isoelectric point) standards is somewhat more difficult to solve, and requires the preparation of mixtures containing large numbers of proteins, differing from each other by single charges. For stained gels, the only requirement is that the proteins all be visible by staining, while for autoradiography or fluorography, radiolabeling is required. It is also desirable to have fluorescently labeled charged standards (or, alternatively, brilliantly dyed standards), so that the isoelectric focusing pH gradient may be monitored during all stages. This is important if anomalies associated with the streaking at the alkaline end of isoelectric focusing gels are to be understood and minimized.

We have previously described the use of progressively carbamylated protein derivatives as internal standards for isoelectric focusing (7). Depending on the protein used, as many as 35 spots extending across a given pH range may be prepared by carbamylation of one protein. Since each of the spots in the carbamylation "train" thus produced can be unambiguously identified by counting from the native spot, the train forms a natural internal coordinate system for isoelectric point measurement in two-dimensional systems.

Here we describe further investigations into the usefulness of internal charge standardization. First, we present approximate measurements of the isoelectric point (in 8 M urea) of selected elements in the creatine phosphokinase (CK) charge train. Next we describe tests of the usefulness of such standards in non-equilibrium [NEPHGE (8) or BASO (9)] two-dimensional systems. The question to be answered is whether a given protein will appear at the same charge standard coordinate in the equilibrium systems (which are usually started at the basic end of the gradient) as in non-equilibrium systems (usually started at the acid end) under a range of running conditions. Lastly, we illustrate some potential use of fluorescently labeled charge standards.

Materials and Methods

Carbamylation was performed using purified rabbit muscle creatine phosphokinase obtained from Sigma. The protein was solubilized in 8 M urea, 1% mercaptoethanol at a concentration of 5 mg/ml. Aliquots were put in glass tubes each containing a small magnetic stirring bar, and were then heated in a boiling water bath for various times, 0, 2, 4, 5, 6, 8, 9, 10, and 12 minutes. After heating, the tubes were put directly on ice to inhibit further modification of the proteins. After running two-dimensional gels of each time point to determine proportions needed to make homologous "train" of spots, a mixture of the intermediate phases of the protein was made. The standard mixture is then frozen down in 50 λ aliquots for later use. For inter-laboratory comparisons, the samples cculd be lyophilized and rehydrated when needed.

The cell lines used for determining measurements on CK charge train were either normal human peripheral lymphocytes or a human lymphoblastoid cell line (GM 607) radioactively labeled with ^{35}S-methionine. The preparation of lymphocytes has been described elsewhere (10). For comparison of the ISO-DALT system (3-4) and the BASO-DALT (9) system, the same sample was used for both, loading 20 λ cell sample plus 2 λ CK standards on the first dimension gels. For the ISO, we ran the sample for 13,000 volt-hours, shown by previous experiments to be an appropriate amount of time for proteins to completely focus. However, small variations in the integrated volt-hours do not appear to move the proteins appreciably once they have found their isoelectric focusing point. In comparison, the BASO run was for various time points: 2000, 3000, 4000, 5000 volt-hours; each time point shifted the more basic proteins toward the alkaline end, so that the separation never appeared to reach equilibrium. After second-dimension electrophoresis, the gels were stained in 0.4% Coomassie Blue and destained in ethanol/5% acetic acid for examination of the "cold" or non-radioactive proteins. After photographing the stained gels, those labeled with 35 S-Methionine were soaked in 2% glycerol before drying on blotting paper were soaked in 2% glycerol before drying on blotting paper, and were then placed against Kodak X-R 2 X-Omat film and exposed for two weeks for

autoradiography.

For fluorescent labeling of proteins, fluorescein isothiocyanate (FITC) was purchased from Sigma. FITC is added to purified proteins after they have been originally stripped of possible bound low molecular weight components on P-6 Biogel 100-200 mesh (Bio-Rad Lab.) The fluorescein is allowed to react with the protein for approximately one minute in the absence of urea. After the reaction has taken place, a 0.1 volume of 1 M ethylenediamene is added to react with any unbound fluorescein, and to prevent excess fluorescein from attaching to unknown foreign substances. The protein-fluorescein complex is passed over another P-6 Biogel 100-200 mesh (Bio-Rad) column, to strip the protein-bound fluorescein from the fluorescein now complexed with ethylenediamene. The protein solution collected was concentrated fivefold on a minicon (Amicon). After isoelectric focusing of hemoglobin-bound fluorescein, sharp fluorescent bands were visible for the α-hemoglobin and β-hemoglobin. For pH measurement, a selected ruled 1/4 inch area, with each band in the center, was cut out and soaked in degassed 8 M urea for five hours. The solution was degassed again before direct pH measurements were made.

Results and Discussion

Our objective is to describe spot positions in two-dimensional gels in a reproducible manner, but we are here concerned only with the first, or isoelectric focusing dimension.

The position of specific proteins in human lymphocyte patterns may be located in reference to creatine phosphokinase charge standards as shown in Figure 1. The difference between charge standard spots -1 and -4 is approximately 0.2 pH units. The small size of the charge standard spots allows localization of nearby pattern spots to approximately 1/5 the distance between spots centers, allowing localization, in the best instances, to between 0.01 and 0.02 pH units.

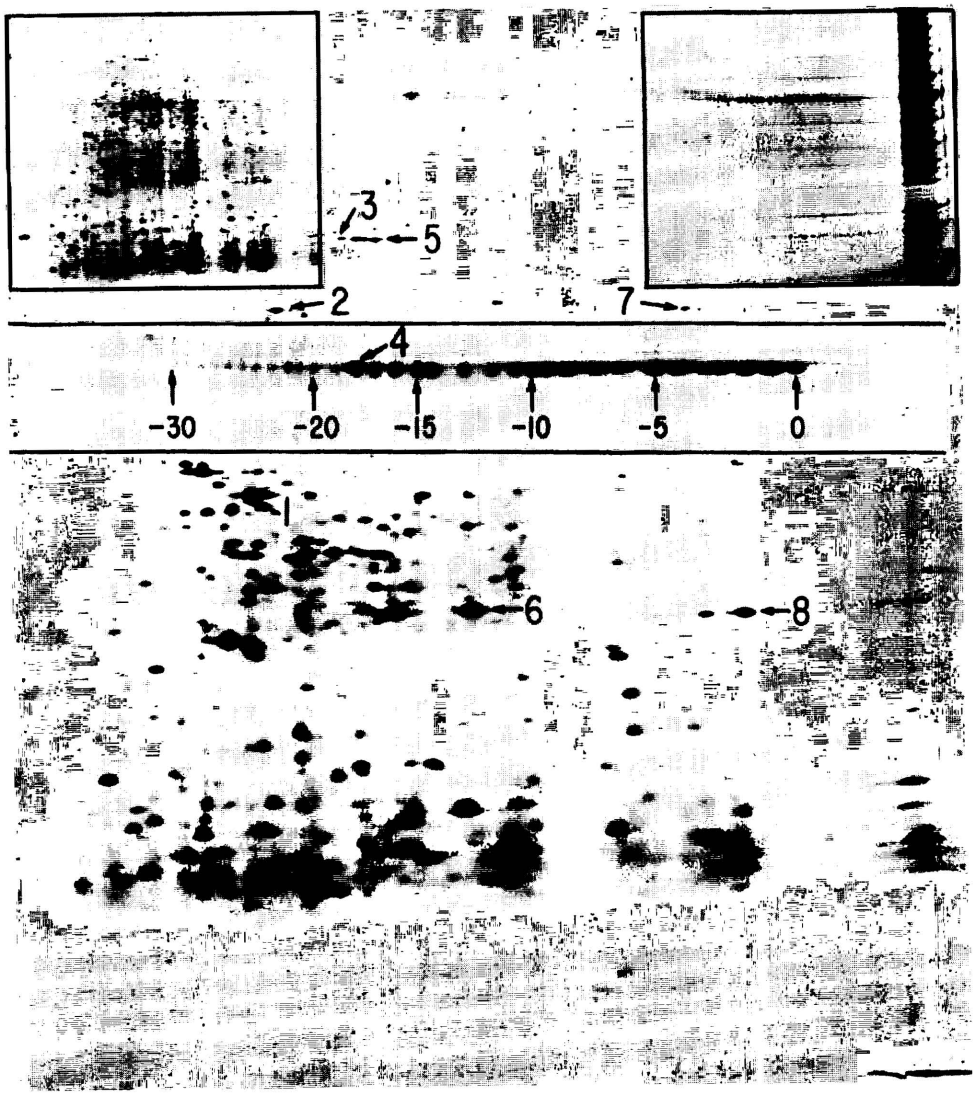

Fig. 1. An autoradiograph of a two-dimensional pattern of human peripheral lymphocytes labeled with ^{35}S-Methionine with insets of the same; Left: the autoradiograph, Right: the Coomassie Blue stained gel co-electrophoresed with CK carbamylation train standards and rat heart molecular weight standards. Partials of the two insets were used to obtain the total picture. Gels are oriented with the acid end to the left, the basic end to the right.
1(pI = -24.0); 2(pI = -22.0); 3(pI = -18.0); 4, actin (pI = -17.5); 5(pI = -17.0); 6(pI = -12.5); 7(pI = -4.0); 8(pI = -2.0).

Different proteins give charge standard trains having different pH ranges, and different interspot distances, depending on the amino acid composition. Proteins having a high charge density give much smaller interspot distances when carbamylated.

Under so-called NEPHGE (8) or BASO (9) conditions, patterns are quite different since the samples are purposefully not run to equilibrium. Here charge standards are useful for identification and localization purposes, as shown in Figure 2, especially at the molecular mass level of the charge standard in the gel. However, they do not appear to indicate the position of a certain pH in the alkaline end of the isoelectric focusing gel. Surprisingly, in the more acid regions of BASO gels, consistent positional data are obtained and actin is found at -17.5 CPK charge units in both ISO and BASO gels.

To investigate fluorescent charge standards we have labeled human hemoglobin by very brief labeling with fluorescein isothiocyanate. The results as seen in fluorescent light are shown in Figures 3 and 4. Since the standard can be seen during focusing we now are able to monitor the rate of approach to final focusing position. Note that the fluorescein labeling is very brief, so that at most only one molecule of dye attached to one hemoglobin subunit, and many are left unmodified. It is then possible to locate both the modified and unmodified subunits with reference to CK charge standards. Unless high pH ampholines are used, the unmodified alpha chain of hemoglobin is rarely seen. However, as shown in Figure 5, one fluorescein molecule moves the beta chain from a position of greater than 0 CK units to a position of -3.5 CK units. Similarly, the alpha chain of hemoglobin is moved approximately the same distance by one fluorescein molecule added, but the proteins are too acid to measure on the CK train. The modified beta hemoglobin is only about 1/20 of the beta hemoglobin present, hence doubly modified beta chains would probably not be visible.

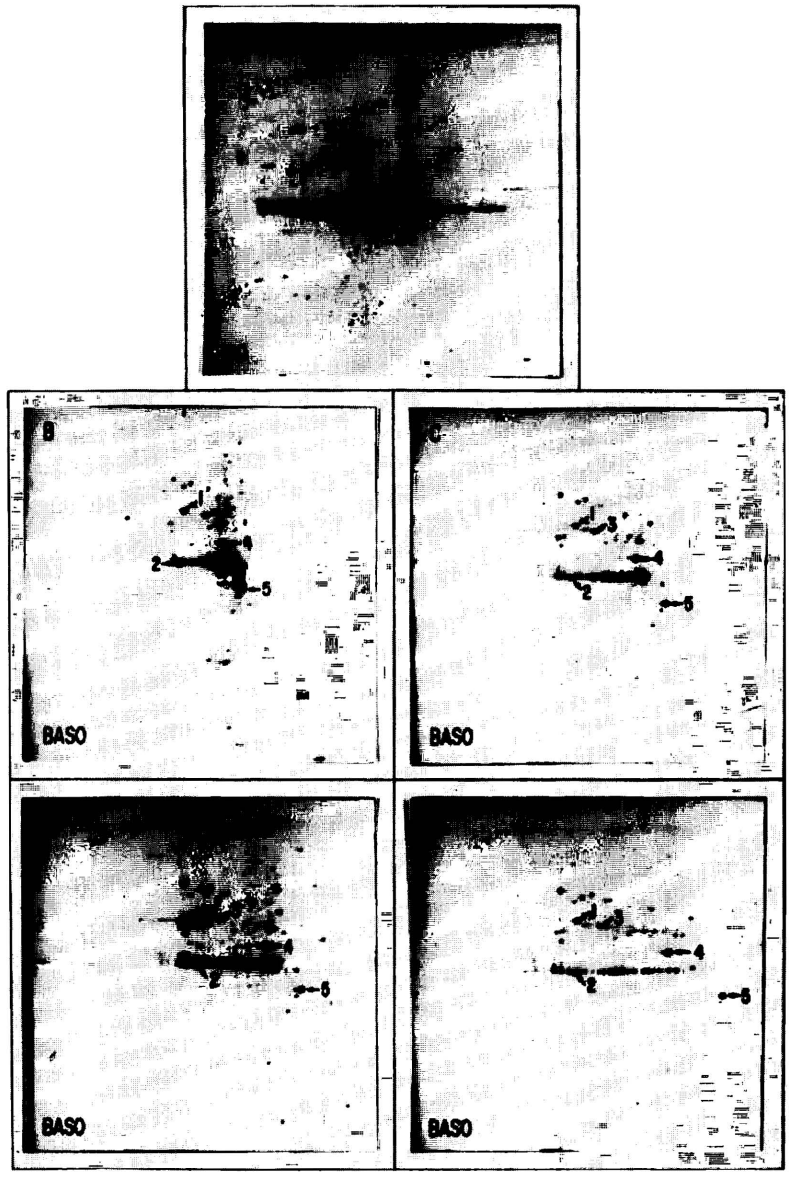

Fig. 2, Two-dimensional electrophoretic patterns of human lymphoblastoid cells (GM 607) showing charge comparisons of the ISO-DALT and BASO-DALT systems. Gels are oriented as in Fig. 1.
1(pI = -17.0); 2, actin (pI = -17.5); 3(pI = -13.0); 4 and 5 present only on BASO gels and varying with volt-hours (V-H). 4[2,000 V-H, pI = -4.0; 3,000 V-H, pI = -2.5; 4,000 V-H, pI = -2.5; 5,000 V-H, pI = -1] 5 is too basic to measure on CK charge train, but varies with V-H.

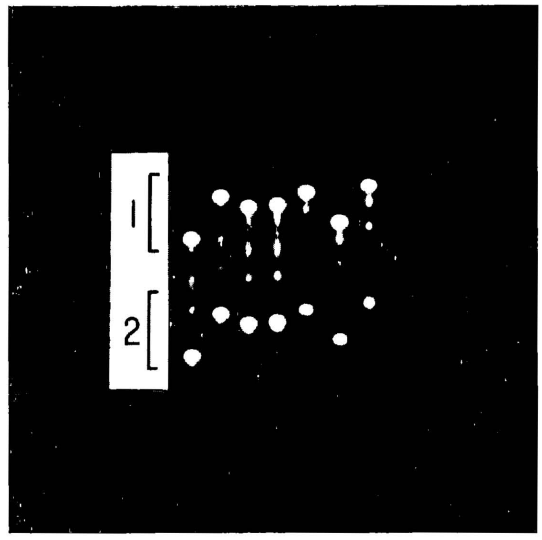

Fig. 3. Isoelectric focusing gels of red blood cell lysate labeled with fluorescein. Photographed with an ultra-violet light source and a green fluorescence filter. Gels are oriented as in Fig. 1.
1, modified alpha - hemoglobin; 2, modified beta - hemoglobin

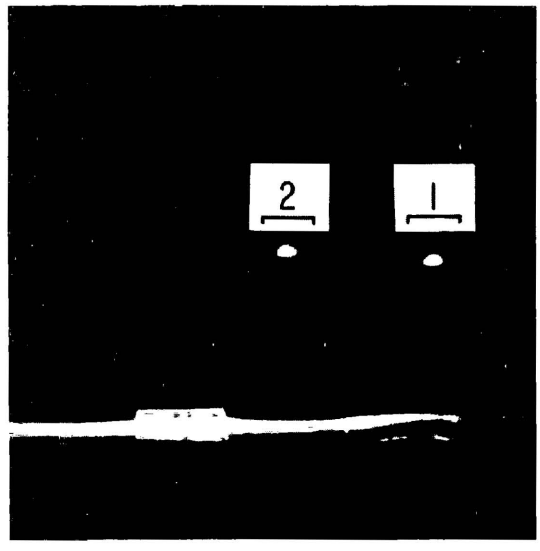

Fig. 4. Two-dimensional electrophoretic pattern of the same gel as in Fig. 3, fluorescein labeled hemoglobin chains. Gels are oriented as in Fig. 1. 1, modified alpha - hemoglobin; 2, modified beta - hemoglobin.

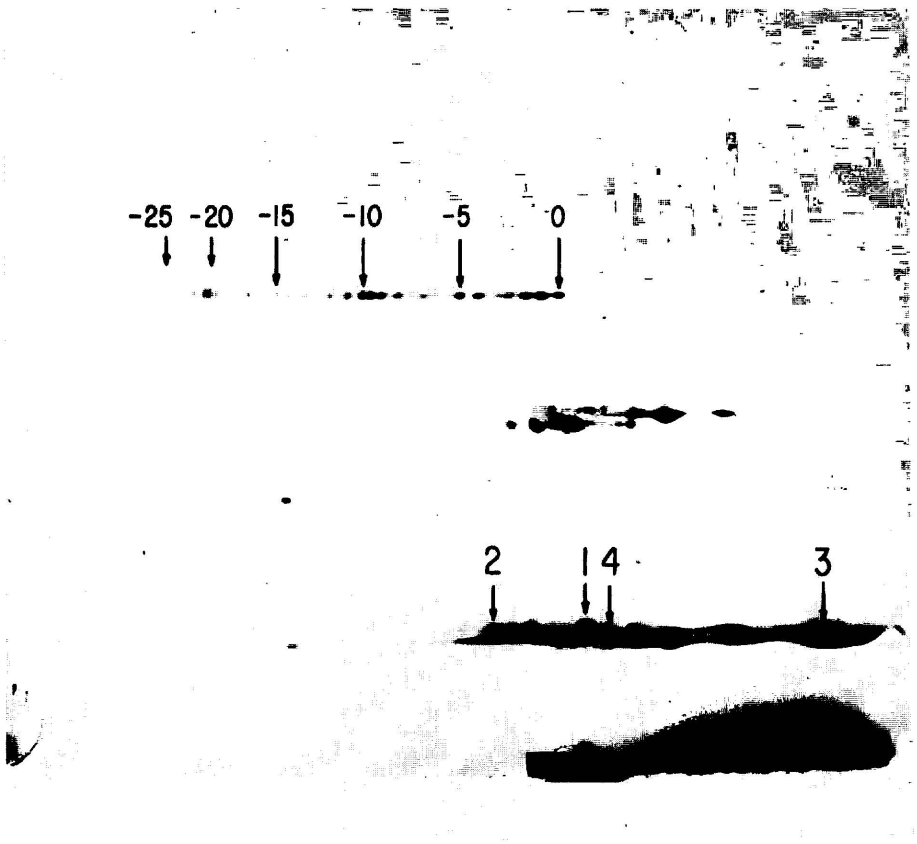

Fig. 5. Two-dimensional pattern of red blood cell lysate stained by Coomassie Blue after fluorescein labeling and using the CK carbamylation train standards. Other RBC lysate proteins are evident which have been identified elsewhere (11). Gels are oriented as in Fig. 1.
1, Native beta - hemoglobin (pI = > 0)
2, Modified beta - hemoglobin (pI = -3.5)
3, Native alpha - hemoglobin (too basic to measure)
4, Modified alpha - hemoglobin (too basic to measure)

The use of CK carbamylation train as standards is useful when the pH range is 5-7. These charge shift markers will reveal the variability between ampholine batches which we have found frequently occur.

Conclusions

By using current methods of 2-D gel spot intercomparisons, we find ourselves virtually without standardization. However, both carbamylated and fluorescein labeled proteins have been shown to be useful internal standards for isoelectric focusing in polyacrylamide gels.

Acknowledgment

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