Journal of Chromatography, 630 (1993) 345-351
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 662

Determination of the number and relative molecular mass of subunits in an oligomeric protein by two-dimensional electrophoresis

Application to the subunit structure analysis of rat liver amidophosphoribosyltransferase

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(First received February 18th, 1992; revised manuscript received October 6th, 1992)

ABSTRACT

To determine simultaneously the relative molecular mass (M_r) of a native oligomeric protein, and the number and M_r of its subunits, a method using two-dimensional electrophoresis was developed. To determine the M_r of a native oligomeric protein, pore gradient gel electrophoresis was performed for the first dimension. Native proteins were dissociated into their subunits by sodium dodecyl sulphate (SDS) in a gel slice, then applied to SDS polyacrylamide gel electrophoresis for the second dimension to determine the M_r of subunits. The advantage, accuracy, limitations and application of the method are discussed.

INTRODUCTION

Most proteins are composed of subunits. The subunit structure plays an important role in exhibiting biological characteristics and in the functional regulation of various oligomeric proteins. The determination of the number and relative molecular mass (M_r) of subunits is therefore essential to understanding the biochemical regulation played by oligomeric proteins.

Although gel chromatography has been widely used to determine the M_r of native oligomeric proteins [1], as it is simple and inexpensive, the results are subject to unavoidable errors due to sample dilution and low resolution capacity. Pore gradient gel electrophoresis (PGGE) based on the concept of gel electrophoresis has been used to determine the M_r of native proteins with a high resolution capacity [2–8]. In PGGE, proteins migrate from the lower to the higher concentration of a polyacrylamide gradient in a slab gel. As the gel pores decrease in size, the migration rate of proteins also decreases. The proteins reach their respective "pore limits" determined by their molecular sizes irrespective of

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ionic charges of protein molecules, and form sharp bands. Although each protein in a band formed at the approximate pore limit continues to migrate very slowly, the relative mobility of each protein becomes constant relative to other proteins. Therefore, the M_r of a native protein of interest can be determined by comparing its migration with those of standard proteins. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) with thiol reagents such as 2-mercaptoethanol (2-ME) or dithiothreitol using a discontinuous buffer system [9,10] has been widely used for the determination of subunit M_r. The mobility of subunits denatured by SDS on PAGE reflects their M_r irrespective of their original charges or conformation [11,12].

Two-dimensional (2D) electrophoresis with a combination of PGGE for the first dimension and SDS-PAGE for the second has been developed to determine simultaneously the M_r of native oligomeric proteins and the number and M_r of their subunits. Oligomeric proteins separated by PGGE were dissociated into subunits by SDS and 2-ME in a gel slice and applied to SDS-PAGE. The M_r of native proteins and the number and M_r of their subunits were established from the positions in the gel compared with those of molecular markers. The accuracy and limitations of this method were tested by using standard oligomeric proteins, the subunit structures of which are known. This method was used to determine the subunit structure of partially purified rat liver ATase (EC 2.1.2.14), which is considered a rate-limiting enzyme in the *de novo* purine synthetic pathway.

EXPERIMENTAL

Materials

Acrylamide-HG, N,N′-methylenebisacrylamide-HG (Bis), SDS, ammonium peroxodisulfhate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), glycerol, 2-ME, glycine, boric acid and EDTA were purchased from Wako (Osaka, Japan) and Tris and 5-phosphoribosyl 1-pyrophosphate (PRPP) from Sigma (St. Louis, MO, USA). Radioactive [^{14}C]glutamine and anti-rabbit [^{125}I]immunoglobulin F(ab') fragment were purchased from Amersham International (Amersham, UK). High-M_r standard proteins for electrophoresis including hog thyroid thyroglobulin (M_r = 669 000, subunit M_r = 330 000), horse spleen ferritin (M_r = 440 000, subunit M_r = 18.5), beef liver catalase (M_r = 232 000, subunit M_r = 36 000), and bovine serum albumin, egg white ovalbumin (M_r = 43 000), bovine carbonic anhydrase (M_r = 232 000, subunit M_r = 60 000), beef heart lactate dehydrogenase (LDH; M_r = 140 000, subunit M_r = 36 000), and bovine serum albumin (M_r = 67 000, monomer), an M_r marker kit for electrophoresis, bromphenol blue, agarose, Sephadex G-25 and a gradient maker were purchased from Pharmacia–LKB Biotechnology (Uppsala, Sweden). A silver staining kit was purchased from Daiichi Pure Chemicals (Tokyo, Japan). A peristaltic pump was purchased from Taiyo Scientific Industrial (Tokyo, Japan). Seven-week-old male Wistar rats and female New Zealand White rabbits weighing 3 kg were purchased from Shizuoka Laboratory Animal Centre (Shizuoka, Japan). A hydroxyapatite column was purchased from Toa Nenryo Kogyo (Tokyo, Japan).

PGGE for the first dimension

Gels were made with a linear gradient from 3 to 20, 22.3, 25 and 30%T[a], respectively; to achieve complete gel transparency [7,8], Bis was added at 2.7%C. The abbreviations %T and %C indicate, respectively, the percentage concentration of acrylamide and Bis per total volume, and the percentage concentration of Bis per %T [13]. The acrylamide solutions at high and low concentrations containing 2.7%C Bis, 0.04% APS, 0.06% TEMED, 22.5 mM Tris–HCl (pH 8.4), 20 mM boric acid, and 0.6 mM EDTA were gradually mixed in a gradient maker and poured into the mould (140 × 140 × 1 mm) assembled with two glass plates and spacers at a constant flow-rate controlled by a peristaltic pump. After polymerization at room temperature, the slab gel was equilibrated with an electrode buffer (90 mM Tris-HCl-80 mM boric acid – 2.5 mM EDTA, pH 8.4) at 70 V for 20 min. Approximately 60 μg of a mixture of five standard proteins were dissolved in 60 μl of electrode buffer with 10% glycerol and applied to the top of the gel. Electrophoresis was performed with the vertical slab gel system at 150 V for 15 h in a cold room.

[a] T = (g acrylamide + g Bis)/100 ml solution.

SDS-PAGE for the second dimension

According to Laemmli's method [9], the lower separating gel (12.5%T, 2.7%C) and the upper stacking gel (3.0%T, 2.7%C) were polymerized in the same mould as used for PGGE. After PGGE, the gel slice was longitudinally excised to a width of 3 mm, then incubated in a small amount of sample buffer containing 62.5 m*M* Tris–HCl (pH 6.8), 2.3% SDS, 5% 2-ME, 10% glycerol and 0.002% bromphenol blue for 1 h at room temperature. To attach the sliced pore gradient gel closely to the upper stacking gel, 0.7% hot agarose solution in the sample buffer was poured onto the top of the upper gel. The gel slice after the incubation with SDS was quickly submerged in this solution before it solidified. The slab gel was set in a vertical system and the electrode buffer containing 25 m*M* Tris–HCl, 192 m*M* glycine and 0.1% SDS was poured into the upper and lower electrode chambers. Proteins in the gel slice and M_r marker proteins were electrophoresed at 20 mA until the tracking dye front migrated to within 1 cm from the gel bottom.

Determination of protein mobility

The gels after electrophoresis were stained with a silver staining kit based on the method of Ohsawa and Ebata [14]. During PGGE, the same protein samples were applied to the individual wells at intervals of 1 h and the migration rate (mm/h) of the protein was calculated as the difference in migration distance between adjacent bands of the same protein. The relative mobility (R_F) on SDS-PAGE was calculated as the ratio of the distance from the top of the separating gel to the densest point in each protein spot relative to the dye front.

Partial purification of rat liver ATase

ATase was partially purified according to the method of Tsuda *et al.* [15] with the following modifications. In brief, purification procedures included ultracentrifugation at 55 000 g for 3 h, heat treatment at 56°C, acid precipitation at pH 5.1, ammonium sulphate fractionation from 30 to 55% and high-performance liquid chromatography through a hydroxyapatite column. ATase was eluted with a linear gradient from 25 to 350 m*M* potassium phosphate buffer with 70 m*M* 2-ME. Fractions containing enzyme activity were desalted through a Sephadex G-25 column equilibrated with the electrode buffer for the first electrophoresis.

Analysis of the subunit structure of rat liver ATase

The desalted rat liver ATase sample was applied to PGGE with 10% glycerol. After the electrophoresis, two gel slices were longitudinally excised to a width of 3 mm. One was cut into 3 × 8 mm sections and the ATase activity in each was assayed as described below. The other slice was applied to the second dimension SDS-PAGE and stained with silver.

Assay of ATase activity

The method for the assay of ATase enzyme activity was the same as reported previously [16]. In principle, PRPP-dependent glutaminase activity was regarded as ATase activity, which was determined by the PRPP-dependent hydrolysis of [^{14}C]glutamine to glutamate.

Preparation of antibodies against two ATase subunits

Partially purified ATase was separated by SDS-PAGE, stained with copper [17] and then M_r 62 000 and 57 000 protein bands were cut out. The gels including each ATase subunit were individually sheared through a 21-gauge needle and conjugated with Freund's adjuvant. The conjugates were injected subcutaneously into female New Zealand White rabbits five times at intervals of 2 weeks. After the fifth injection, blood was drawn and the serum containing antibodies for each ATase subunit was separated. Western blotting was performed by a standard method [18] using a radioactive second antibody.

RESULTS AND DISCUSSION

The standard proteins, including thyroglobulin, ferritin, catalase, LDH and albumin, were applied to PGGE. During the electrophoresis, the migration rate (per unit time) of the standard proteins decreased gradually and reached a constant value as their respective pore limits were approached (Fig. 1). The proteins with a larger M_r reached their pore limits relatively sooner. As even the smallest protein, albumin, reaches its pore limit after 10 h at 150 V and an electrophoretic time equivalent to about 2000 V h has been recommended previously [7], PGGE in this study was performed at 150 V for 15 h. After PGGE, each standard protein was concen-

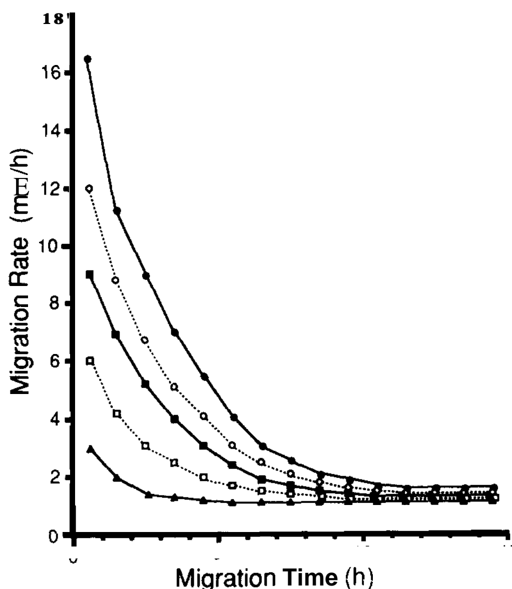

Fig. 1. Change of the migration rate of standard proteins during PGGE with a linear gradient from 3 to 30%T. A = Thyroglobulin; □ = ferritin; ■ = catalase; 0 = LDH; ● = albumin.

trated in a sharp band in the gel as shown in Fig. 2. The calibration graph of migration distance *versus* log M_r was linear with a correlation coefficient of -0.9996 in the M_r range from 140 000 of LDH to 669 000 of thyroglobulin. However, the migration at 10 cm of albumin in the gradient gel from 3 to 22.3%T was 15% more than that expected at 8.7 cm. These results suggest that the calibration graph is actually sigmoidal and the range obeying a linear relationship on a semi-logarithmic scale can be used for accurate M_r determination by PGGE and gel filtration [1]. Indeed, the relationship between the migration distance and log M_r for all standard proteins including albumin is linear in the gradient gel from 3 to 30%T (data not shown). Thus, the linear range is dependent on the gel concentration. The migration distance of an oligomeric protein in PGGE is dependent on its molecular size and shape. Because all standard protein molecules used in this study were globular, as are the majority of oligomeric proteins, the relationship between migration distance and log M_r of standard proteins was linear.

Ferritin. catalase. LDH and albumin in a gel slice

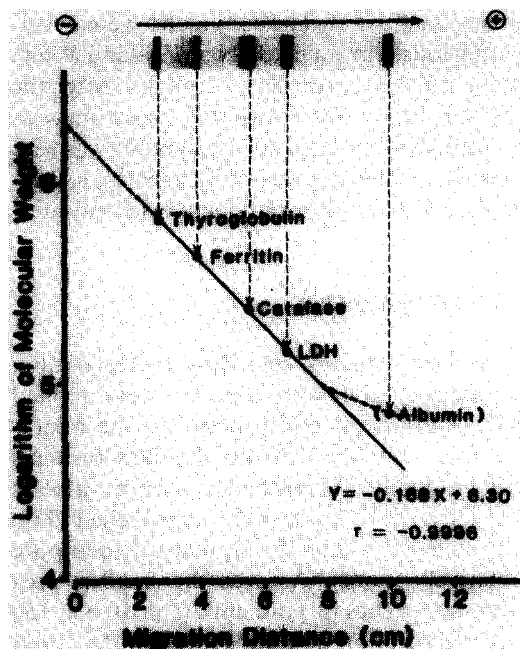

Fig. 2. Plots of the migration distance of the standard proteins against log M_r after PGGE with a linear gradient from 3 to 22.3%T.

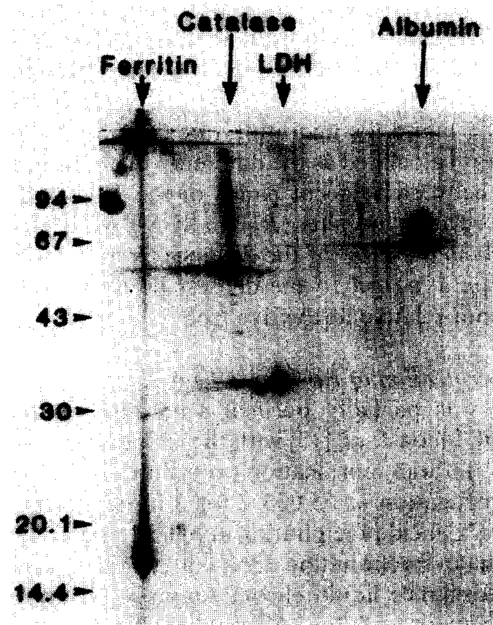

Fig. 3. Two-dimensional electrophoresis of four standard proteins with a combination of PGGE and SDS-PAGE. The positions of the M_r marker proteins are shown on the left (M_r values $\times 10^{-3}$).

TABLE I

COMPARISON BETWEEN CALCULATED AND ESTABLISHED VALUES: NATIVE PROTEIN M_r OBTAINED BY PGGE FROM 3 TO 23.3%T

Protein	Measured M_r	True M_r	Deviation (%)
Thyroglobulin	674 000	669 000	+0.7
Ferritin	442 000	440 000	+0.5
Catalase	226 000	232 000	−2.6
LDH	143 000	140 000	+2.1
Albumin	(65 000)[a]	67 000	−3.0

[a] Data obtained by the gradient gel from 3 to 30%T.

TABLE III

COMPARISON BETWEEN CALCULATED AND ESTABLISHED VALUES: NO. OF SUBUNITS

Protein	Calculated No. of subunits	True No. of subunits	Judgement
Thyroglobulin	2.1 (2)	2	Correct
Ferritin	24.6 (25)	24	Incorrect
Catalase	3.6 (4)	4	Correct
LDH	4.1 (4)	4	Correct
Albumin	1.0 (1)	1	Correct

after separation by PGGE were applied to SDS-PAGE. The silver-stained gel after 2D electrophoresis is shown in Fig. 3. Ferritin gave two protein spots at M_r 220 000 (a half unit) and 18 500 (a true subunit) because of incomplete dissociation. Catalase, LDH and albumin each migrated as one spot. Because the ferritin in the gel slice was completely separated into its subunits by boiling with SDS for 5 min before the second electrophoresis, the gel slice after PGGE should be boiled so long as the peptide bonds of proteins are not cleaved.

In addition to the M_r of native proteins, the number and M_r of their subunits were determined and are summarized in Tables I-III. The M_r valves determined for either native proteins by PGGE or their subunits by SDS-PAGE deviated from the established M_r by less than 3 and 4%, respectively. Because the M_r of albumin is too small to be determined using a gradient gel from 3 to 22.3%T, it was obtained from migration in a gel containing 3–30%T. Also, because the subunit of thyroglobulin hardly entered the separating gel at 12.5%T, its M_r was determined as 3 17 000 by SDS-PAGE at 6%T. The calculated numbers of subunits for thyroglobulin, ferritin, catalase, LDH, and albumin were 2, 25, 4, 4 and 1, respectively. Because the true numbers of subunits are 2, 24, 4, 4 and 1, respectively, the calculated values were precise except for ferritin. Although the deviations in the M_r determination for both native ferritin and its subunit were less than 3%, the calculated number of its subunits was not correct. Therefore, the accuracy of the calculated number of subunits depends on the number of subunits itself, the number of different subunits and the experimental error. According to the calculation from the deviations of M_r determined by PGGE and SDS-PAGE, the proposed method can precisely determine the number of subunits in an oligomeric protein when it is $\leqslant 8$.

Fig. 4 shows the results of the analysis of the

TABLE II

COMPARISON BETWEEN CALCULATED AND ESTABLISHED VALUES: SUBUNIT M_r OBTAINED BY SDS-PAGE AT 12.5%T

Protein	R_F	Measured subunit M_r	True subunit M_r	Deviation (%)
Thyroglobulin	0	(317 000)	333 000	−3.9
Ferritin	0.732	18 000	18 500	−2.7
Catalase	0.267	62 000	60 000	+ 3.3
LDH	0.484	35 000	36 000	−2.8
Albumin	0.232	68 000	67 000	+ 1.5

[b] Data obtained by SDS-PAGE at 6%T.

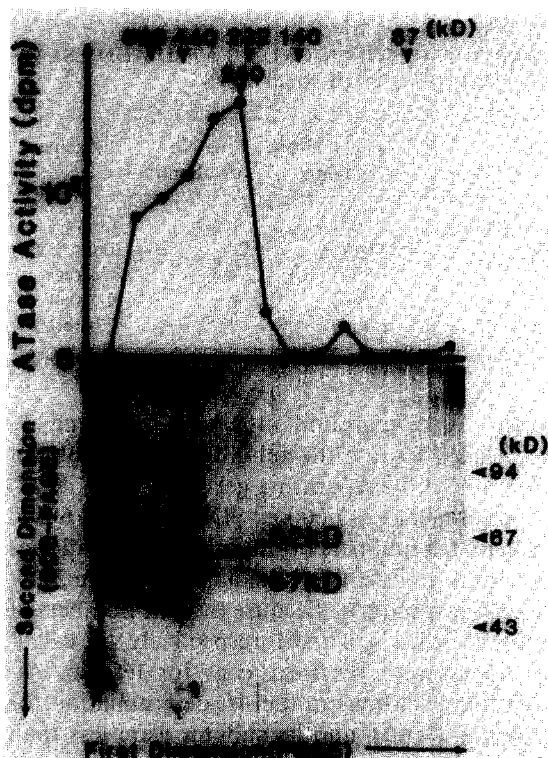

Fig. 4. Analysis of ATase subunit structure. After PGGE, the gel was sliced into two. One section was used to assay ATase activity (upper half). The other section was applied to 2D electrophoresis and silver stained (lower half). The arrowheads indicate M_r 62 000 and 57 000 subunits of ATase, respectively. The positions of standard proteins in the first-dimension PGGE those of M_r marker proteins in the second-dimension SDS-PAGE are shown at the top and on the right, respectively (M_r values $\times 10^{-3}$). The native M_r (240 000) of ATase obtained by PGGE was similar to the values given by gel chromatography and sucrose density gradient centrifugation (data not shown). There was a single protein band corresponding to M_r 240 000 on PGGE and this protein was dissociated into M_r 62 000 and 57 000 protein bands. Considering the sensitivity of silver staining, the amount of ATase protein and the enzyme activity are comparable. However, ATase activity is very labile, especially at its pore limit where molecular jamming, aggregation or shearing in the gel network and the subsequent heating due to increased electric resistance may occur. Therefore, the enzyme activity is not completely proportional to the staining intensity of the M_r 62 000 and 57 000 protein bands. The lower a gel concentration is, the more directly proportional is the relationship between the activity and the staining intensity. kD = kilo dalton.

ATase subunit structure. A peak of ATase activity was detected in the gel piece and its pore limit corresponded to the size of M_r 240 000 globular proteins.

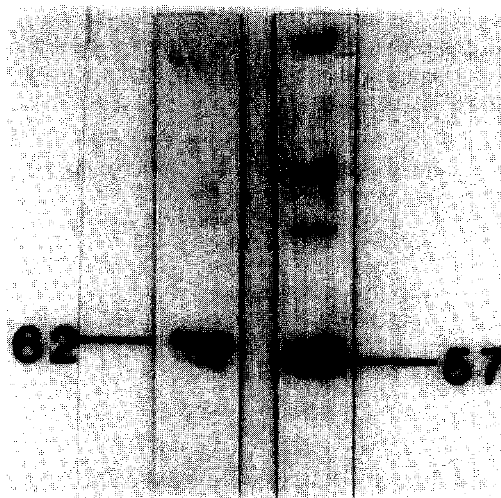

Fig. 5. Western blotting of M_r 62 000 and 57 000 subunits of ATase. Although both anti-M_r 62 000 (left) and 57 000 (right) subunit antibodies have a slight cross-reactivity with each other, the antigenicity of each is apparently different.

The protein at this point was shown by 2D electrophoresis to be composed of M_r 62 000 and 57 000 subunits. These results suggested that ATase is an M_r 240 000 heterotetrameric protein with two kinds of subunits. To ascertain whether or not the M_r 57 000 subunit of ATase was the degradation product of the M_r 62 000 subunit, the difference in antigenicity of the two subunits was examined by Western blotting (Fig. 5). Because the antigenicities of the M_r 62 000 and 57 000 subunits were different, it is suggested that the M_r 57 000 subunit is not the degradation product of the M_r62 000 subunit and that ATase is a heterotetrameric protein. However, both anti-M_r 62 000 and anti-M, 57 000 subunit antibodies had a little cross-reactivity to the other subunit, and 240 000 measured as the native M_r of ATase is nearly equal to 248 000, that is, the sum of four M_r 62 000 subunits, considering of the deviation of this technique. Therefore, a more detailed analysis of ATase subunits including N-terminal sequencing, HPLC mapping of cleavage fragments and cDNA cloning, etc., is necessary to obtain definitive evidence that the M_r 57 000 protein is not derived from the M_r 62 000 subunit.

Because PGGE was performed under non-denaturing condition, the biological activities of most proteins were conserved, as shown for ATase.

Moreover, because the proteins in the sample were concentrated both by PGGE and SDS-PAGE, a high degree of resolution was attained. This method is therefore useful even for very small amounts of the impure protein samples. In fact, 1 μg of rat liver ATase in an impure sample was determined by this 2D electrophoresis. The method is also applicable to basic proteins such as histone with a p*I* above the pH 8.4 of the electrode buffer in PGGE, by exchanging the anode for a cathode (data not shown).

In conclusion, the analysis of the subunit structure of an oligomeric protein by the present method is very useful as it has a high resolution capacity when its limit of accuracy is properly considered.

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