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Anomalous behaviour of a protein during SDS/PAGE corrected by chemical modification of carboxylic groups

André MATAGNE, Bernard JORIS and Jean-Marie FRÈRE*
Laboratoire d'Enzymologie, Université de Liège, Institut de Chimie, B6, B-4000 Sart-Tilman (Liège 1), Belgium

The 29000-M_r *Actinomadura* R39 β-lactamase exhibited a remarkably low electrophoretic mobility on SDS/PAGE, yielding an M_r value almost twice that computed from the corresponding gene sequence. We showed that chemical modification of the carboxylic groups of glutamic acid and aspartic acid residues restored a normal electrophoretic mobility and that the anomalous behaviour of that protein on SDS/PAGE was due to its very large negative charge at neutral pH. We also compared the behaviour of the same enzyme on gel filtration in the presence of SDS with those of other class A β-lactamases (M_r approx. 30000). These experiments suggested that the very low electrophoretic mobility of the *Actinomadura* R39 β-lactamase upon SDS/PAGE was more probably due to a low degree of SDS binding rather than to an unusual shape of the SDS–protein complex.

INTRODUCTION

Determination of the M_r values of polypeptides by using SDS/PAGE was first introduced, empirically, by Shapiro *et al.* (1967), and then developed and enlarged by Weber & Osborn (1969) and Dunker & Rueckert (1969). Since that time, that simple technique has emerged as the most popular, easiest and cheapest method for determining the M_r values of protein subunits (for a complete review of the method see See & Jackowski, 1990). However, although it is clearly established that, under appropriate conditions, most reduced polypeptides bind SDS in a constant weight ratio (1.4 g of SDS/g of polypeptide) (Reynolds & Tanford, 1970*a*), the molecular explanation for the ability of widely different proteins to form complexes that contain approximately the same amount of detergent on a gram-to-gram basis remains rather obscure and a subject of controversy (Reynolds & Tanford, 1970*b*; Mattice *et al.*, 1976; See & Jackowski, 1990). As the method relies on the assumption that the electrophoretic mobility on SDS/PAGE depends only on the M_r of a polypeptide, which implies that all such compounds share identical charge/mass ratios and shapes when complexed with SDS, the empirical nature of this technique should be kept in mind.

Several problems can be encountered in M_r determinations. Indeed the method appears to yield anomalous values for glycoproteins (Segrest *et al.*, 1971; Ahmed & Furth, 1990), very hydrophobic proteins (Bayreuther *et al.*, 1980), very basic proteins (Panyim & Chalkley, 1971) or very acidic proteins (Burton *et al.*, 1981; Kaufman *et al.*, 1984; Georges & Mushynski, 1987) and low-M_r polypeptides (Hames, 1981).

In the present paper we analyse the anomalous behaviour of the β-lactamase from *Actinomadura* R39 on SDS/PAGE. Attempts to measure the size of this protein by direct methods yielded conflicting M_r values ranging from 15000 (Duez *et al.*, 1982) to 55000 (Houba *et al.*, 1989). On the basis of the nucleotide sequence of the gene, an M_r value of 29270 was calculated (Houba *et al.*, 1989). Here we demonstrate that the surprisingly low mobility of that protein on SDS/PAGE (which gave rise to the highest M_r value of 55000) is due to its large negative charge at neutral pH (pI < 4; Matagne *et al.*, 1991) and that chemical modification (hydrazination) of the carboxylic groups of glutamic acid and aspartic acid residues restores a normal electrophoretic mobility.

MATERIALS AND METHODS

Proteins

β-Lactamases. *Actinomadura* R39, *Bacillus licheniformis* 749/C, *Streptomyces albus* G and *Streptomyces cacaoi* β-lactamase preparations were the same as those used for the study of the catalytic properties of these enzymes (Matagne *et al.*, 1990).

Electrophoresis standards for M_r determination. Standards for M_r determination by SDS/PAGE were low-M_r standards from Bio-Rad Laboratories, containing lysozyme (M_r 14400), soya-bean trypsin inhibitor (M_r 21500), carbonic anhydrase (M_r 31000), ovalbumin (M_r 43000), BSA (M_r 66000) and phosphorylase *b* (M_r 97000).

Electrophoresis and M_r determinations

PAGE under non-denaturing conditions at pH 8.8 or in the presence of 0.1% SDS at pH 8.8 was performed in a Bio-Rad Mini-Protean II dual slab cell as described in the accompanying instruction manual.

Denaturing conditions. M_r values were determined by heating the proteins (unknown and standards) at 100 °C for a few minutes in the presence of 1% SDS and 1 mM-dithiothreitol, then running the samples on a 12% (w/v) polyacrylamide gel in the presence of 0.1% SDS and measuring their relative mobilities as described in See & Jackowski (1990).

Non-denaturing conditions. M_r values were determined as described by Thorun & Maurer (1971). The migrations of the protein to be analysed and of four reference proteins [myoglobin (M_r 17000) and carbonic anhydrase (M_r 29000) from Serva and ovalbumin (M_r 43000) and BSA (M_r 66000, 132000 and 199000) from Sigma Chemical Co.] were determined in gels prepared with concentrations ranging from 8 to 12% (w/v) polyacrylamide.

Gel chromatography and M_r determinations

A Superdex 200 Hi Load 16/60 column connected to a Pharmacia f.p.l.c. system was used. Samples, prepared as for SDS/PAGE, were filtered through the 110 ml Superdex 200 column in 100 mM-sodium phosphate buffer, pH 7, containing 0.1% SDS. Their distribution coefficients ($K_{av.}$) were compared with those of four standard proteins (lysozyme, carbonic anhydrase, ovalbumin and BSA) to determine their M_r values.

* To whom correspondence should be addressed.

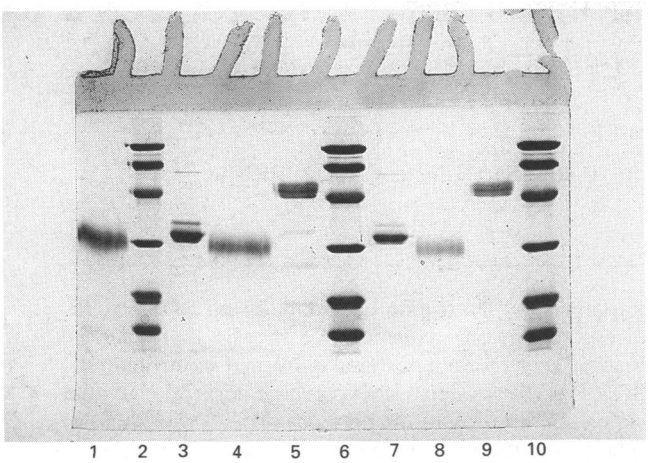

Fig. 1. SDS/PAGE of class A β-lactamases

The gel contained 12% acrylamide. Lanes 2, 6 and 10: M_r standards (phosphorylase *b*, BSA, ovalbumin, carbonic anhydrase, soya-bean trypsin inhibitor and lysozyme). Lanes 3 and 7: *S. albus* G β-lactamase. Lanes 5 and 9: unmodified *Actinomadura* R39 β-lactamase. Lanes 1, 4 and 8: hydrazine-modified *Actinomadura* R39 β-lactamase.

Chemical modificatin of glutamic acid and aspartic acid residues

The *Actinomadura* R39 β-lactamase (0.5 mg) dissolved in 0.2 ml of 50 mM-sodium phosphate buffer, pH 7, was mixed with 1.7 ml of 8 M-urea containing 1 M-hydrazine and adjusted to pH 4.5 with HCl. The solution was stirred during 2 h at room temperature in the presence of 0.1 M-1-dimethylaminopropyl-3-ethylcarbodi-imide and the pH was maintained below 4.7.

The pK_a of benzoic acid hydrazide (Janssen, Geel, Belgium) was determined by titration with 1 M-HCl. A value of 3 ± 0.1 was obtained, in good agreement with those found for *p*-nitrobenzhydrazide (2.77; Paulsen & Stoye, 1970) and acetic acid hydrazide and glycylhydrazide (3.24 and 2.38 respectively; Lindegren & Niemann, 1949).

RESULTS AND DISCUSSION

Electrophoresis

Electrophoresis of the *Actinomadura* R39 β-lactamase, performed on a 12% polyacrylamide gel in the presence of 0.1% SDS, reproducibly yielded an M_r of 55000 ± 3000 (Fig. 1, lanes 5 and 9). The very low electrophoretic mobility of this protein was particularly striking when compared with that of the *S. albus* G β-lactamase, a protein of very similar M_r (Fig. 1, lanes 3 and 7). On the basis of the gene sequence (Dehottay *et al.*, 1987), an M_r of 29500 was computed for the latter protein and an apparent value of 36000 ± 2000 was deduced from its electrophoretic mobility on SDS/PAGE. Two other class A β-lactamases (Ambler, 1980), from *S. cacaoi* and *B. licheniformis*, characterized also by M_r values very close to 30000 (based on their known sequences), were run on SDS/PAGE and appeared to behave 'normally' (Table 1). Thus the anomalous behaviour of the *Actinomadura* R39 β-lactamase did not appear to be a common feature of the 30000-M_r class A β-lactamases.

Non-denaturing PAGE was also performed with the *Actinomadura* R39 β-lactamase, with different concentrations of acrylamide (Thorun & Maurer, 1971), and yielded an M_r value of 35000 ± 4000, thus much closer to the expected value.

The anomalous behaviour of the *Actinomadura* R39 β-lactamase under denaturing conditions could not be attributed to the presence of a carbohydrate moiety. Indeed, a phenol/H_2SO_4 test (Dubois *et al.*, 1956) was negative. However, this enzyme is known to be very negatively charged at neutral pH (pI < 4; Matagne *et al.*, 1991). Indeed, the amino acid composition of the *Actinomadura* R39 β-lactamase is highly asymmetric and atypical [24% and 8% of acidic and basic residues respectively compared with 12% and 14% for an average protein (Dayhoff *et al.* (1978)]. According to its amino acid sequence (Houba *et al.*, 1989), the protein contains 41 glutamic acid and 24 aspartic acid residues compared with two lysine, 14 arginine and five histidine residues. On that basis, a net charge of −47 to −49 can be expected at pH 7. Thus the low electrophoretic mobility of this protein could surprisingly be due to its very high negative net charge. To check this hypothesis, we chemically modified the carboxylic groups of glutamic acid and aspartic acid residues

Table 1. M_r values of different class A β-lactamases as measured with the help of various techniques

Source of β-lactamase	M_r value			
	PAGE	SDS/PAGE	SDS/gel filtration	Deduced from sequence*
S. albus G	31500 ± 200†	36000 ± 2000	38000 ± 2000 ($K_{av.}$ = 0.19)	~ 29500‡
B. licheniformis	–	31500 ± 1000	37000 ± 2000 ($K_{av.}$ = 0.20)	~ 29500§
S. cacaoi	–	28500 ± 1000	–	~ 30200‖
Actinomadura R39	35000 ± 4000	55000 ± 3000	42000 ± 2000 ($K_{av.}$ = 0.17)	~ 29000¶
Actinomadura R39 (chemically modified enzyme)	–	31500 ± 500	30000 ± 2000 ($K_{av.}$ = 0.25)	~ 29000

* No accurate M_r values can be quoted in this column since all these proteins exhibit ragged *N*-termini (Matagne *et al.*, 1991). Generally, 10–15 residues can be missing in the shortest form.

† From Duez *et al.* (1981).

‡ From Dehottay *et al.* (1987).

§ From Ambler (1980).

‖ From Lenzini *et al.* (1988).

¶ From Houba *et al.* (1989).

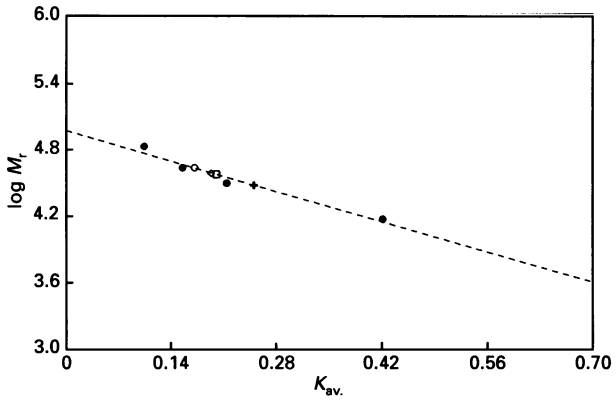

Fig. 2. Calibration curve of log M_r versus distribution coefficients for a gel-filtration experiment through a Superdex 200 column in the presence of SDS

The standard proteins (●) were, in order of decreasing M_r, BSA, ovalbumin, carbonic anhydrase and lysozyme. The studied proteins were the class A β-lactamases from *S. albus* G (◇), *B. licheniformis* (□) and *Actinomadura* R39 (○) and the modified *Actinomadura* R39 enzyme (+).

with hydrazine (see the Materials and methods section), transforming them into hydrazide groups. These monoacylhydrazines are weakly basic (Paulsen & Stoye, 1970; see the Materials and methods section) and therefore are neutral under our experimental conditions. Thus we drastically modified the net charge of the protein. Indeed, if all the carboxylic groups were modified, the net charge changed to +16 to +18. As shown in Fig. 1 (lanes 1, 4 and 8), reaction of the *Actinomadura* R39 β-lactamase with hydrazine in the presence of a water-soluble carbodi-imide yielded a modified protein that upon SDS/PAGE exhibited an electrophoretic mobility characteristic of a 31500 ± 500-M_r polypeptide. This clearly demonstrated that the high intrinsic negative charge of the *Actinomadura* R39 β-lactamase was responsible for its remarkably low electrophoretic mobility on SDS/PAGE.

If it seemed very probable that the numerous negative charges of the protein somehow interfered with the binding of the SDS, we could not decide if its anomalous electrophoretic mobility was due to a lower-than-average degree of SDS binding or to an atypical shape of the SDS–protein complex. To investigate this problem further, we studied the behaviour of the *Actinomadura* R39 β-lactamase upon gel chromatography in the presence of SDS.

Gel chromatography

Fish *et al.* (1970) pointed out that gel chromatography can be used as a substitute for other hydrodynamic measurements in the determination of the gross conformation of proteins in SDS. Non-modified and chemically modified *Actinomadura* R39 β-lactamase preparations were filtered through a Superdex 200 column and their distribution coefficient ($K_{av.}$) values were calculated and compared with those of other class A β-lactamases (M_r approx. 30000) (Table 1 and Fig. 2). Firstly it appeared that the three β-lactamases (*Actinomadura* R39, *S. albus* G and *B. licheniformis*) exhibited approximately the same distribution-coefficient values and consequently the same size and shape in SDS. It seemed thus very likely that the numerous negative charges of the protein hindered an optimal fixation of SDS and that *Actinomadura* R39 β-lactamase bound much less SDS per gram than do most polypeptides. On that basis, the remarkably low electrophoretic mobility of the *Actinomadura* R39 β-lactamase would then be essentially due to a low degree of SDS binding rather than to an anomalous shape of the SDS–protein complex.

Compared with standard proteins, the computed M_r values of the three β-lactamases tested were around 40000 (Fig. 2), which is significantly higher than the values deduced from their complete amino acid sequences (M_r approx. 30000). Surprisingly, the distribution coefficient of the modified *Actinomadura* R39 β-lactamase was higher (Fig. 2) than those obtained with the non-modified enzyme and other β-lactamases and its deduced M_r was about 30000, which is fairly close to the value computed from the sequence. These latter observations, which might suggest a somewhat higher-than-usual gyration radius for the three SDS–β-lactamase complexes, could not be interpreted on the basis of the experiments described here.

CONCLUSIONS

The 29000-M_r *Actinomadura* R39 β-lactamase exhibited a remarkably low electrophoretic mobility on SDS/PAGE. The M_r value deduced from these experiments was almost twice the value computed from its complete amino acid sequence.

This anomalous behaviour on SDS/PAGE appeared to be due to the large negative charge of this enzyme, since hydrazination of the carboxylic groups of glutamic acid and aspartic acid residues restored a normal electrophoretic mobility. If the modified enzyme acquired a large (16 to 18) net positive charge, that did not affect its behaviour. On the basis of gel-chromatography experiments in the presence of SDS, we suggest that the low electrophoretic mobility of the *Actinomadura* R39 β-lactamase was mainly due to a particularly poor binding of the detergent rather than to an unusual shape of the SDS–protein complex. This is in agreement with the results obtained by Nelson (1971), who proposed that the very low amount of SDS fixed by non-denatured pepsin at neutral pH was due to the large negative net charge of that protein.

In the gel-filtration experiment, the three studied β-lactamases again exhibited a behaviour characteristic of a larger-than-expected size. For the *S. albus* G β-lactamase the values deduced from SDS/gel chromatography and SDS/PAGE were in reasonable agreement, but for the *B. licheniformis* enzyme the value from SDS/PAGE was much closer to the real one. No satisfactory explanation could be found for those observations.

We here emphasize that approximate M_r determination of polypeptides by SDS/PAGE is obviously a very useful method, but the results should always be considered with care, since various problems can be encountered. One of these is related to the low mobility of very acidic proteins.

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