Anomalous Migration of Leghaemoglobin on Sodium Dodecyl Sulphate/Polyacrylamide-Gel Electrophoresis

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The estimate of the molecular weight of leghaemoglobin by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis is about 20% too low. This is due to an anomalously high limiting relative mobility. Leghaemoglobin binds 1.4g of sodium dodecyl sulphate/g of protein with a concomitant decrease in the helical content from 71–72% to 49–51%.

The error in determination of protein molecular weights by SDS/polyacrylamide-gel electrophoresis is usually not more than 5% (Dunker & Rueckert, 1969; Weber & Osborn, 1969). The theoretical background of the method is unclear, and the constant binding ratio, 1.4g of SDS/g of protein (Pitt-Rivers & Impiombato, 1968; Reynolds & Tanford, 1970), has not been explained. Some proteins do not migrate according to molecular weight, e.g. ribonuclease, collagen, histones, some glycoproteins, maleylated proteins and gliadin (Dunker & Rueckert, 1969; Furthmayr & Timpl, 1971; Panyim & Chalkley, 1971; Segrest *et al.*, 1971; Banker & Cotman, 1972; Tung & Knight, 1972; Shirahama *et al.*, 1974; Hamauzu *et al.*, 1975). In contrast with these proteins, which have electrophoretic mobilities that are too low for their molecular weights, leghaemoglobin migrates too fast. The abnormal mobility of leghaemoglobin is particularly unexpected because other globins are reliable molecular-weight standards.

Experimental

SDS (Fluka A.G., Buchs, Switzerland) and urea (Merck, Darmstadt, W. Germany) were recrystallized twice from ethanol. The proteins used as standards were from Sigma Chemical Co., St. Louis, MO, U.S.A., and leghaemoglobins were purified as described previously (Lehtovaara, 1977).

Electrophoresis was performed in the presence of 0.1% (w/v) SDS at pH7.2 (Weber & Osborn, 1969) in 6mm×70mm gels. The ratio of cross-linker to acrylamide was always 1:37. The samples were applied in 10mM-sodium phosphate buffer, pH7.2, containing 1% (w/v) SDS and 1% (w/v) 2-mercaptoethanol, and they were heated for 2min in a 100°C water bath before application of 5μg of each protein per gel. After electrophoresis at room temperature (23°C) at 8mA/gel the dye was marked

Abbreviation used: SDS, sodium dodecyl sulphate.

with copper wire and the gels were stained for 2–12h with 0.25% (w/v) Coomassie Brilliant Blue in methanol/acetic acid/water (5:1:5, by vol.). Destaining at 37°C in 25% (v/v) methanol/7% (v/v) acetic acid was accelerated in rotating tubes containing Amberlite IRA-400 AG resin. The mobility of each sample was taken as the mean of the results from five to twelve gels for each acrylamide concentration (5–15%). The reproducibility was always within ±2%.

Electrophoresis in the presence of urea was performed as described by Swank & Munkres (1971), and the additional standards used were the CNBr peptides from horse cytochrome (a gift from Dr. Ritva Soininen).

The binding of SDS was followed by equilibrium dialysis at 28°C against 50mM-sodium phosphate buffer, pH7.0, containing 0.02% (w/v) NaN_3 and 4mM-SDS. The protein concentrations were determined in an amino acid analyser (Beckman 120 B) from hydrolysed samples, and the SDS concentrations as described by Takagi *et al.* (1975).

The circular-dichroic spectra were recorded with a Cary 61 spectropolarimeter calibrated with (+)-camphorsulphonic acid (Eastman Kodak, Rochester, NY, U.S.A.). The Millipore-filtered samples were about 100μM in 50mM-sodium phosphate buffer, pH7.0, containing 0, 4mM- or 40mM-SDS. Exact protein concentrations were determined as above. The light-path of the cell was 0.1mm, and the cell holder was thermostatically controlled at 28°C. The mean residue weights were calculated from amino acid-sequence data. Calculation of the helical contents was by the method of Chen *et al.* (1972).

Results and Discussion

Leghaemoglobin migrated atypically fast in the electrophoretic systems of Weber & Osborn (1969) and Swank & Munkres (1971), the apparent mol.wts. being 13000±200 and 12000 respectively. The latter
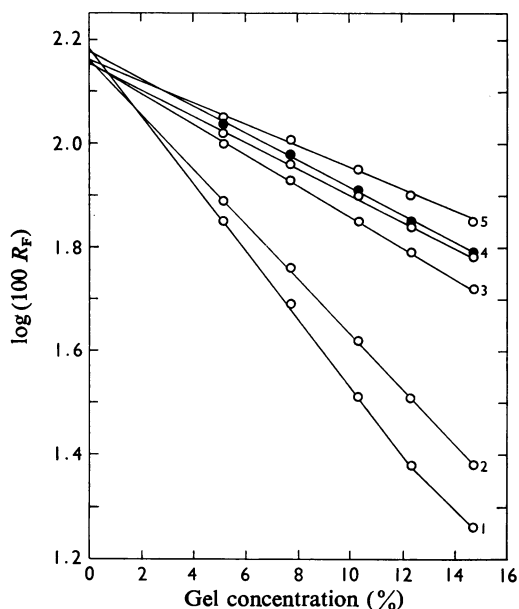
Fig. 1. *Plot of log(relative mobility) against gel concentration*

The standard proteins (○) are: 1, ovalbumin (mol.wt. 43000); 2, pepsin (mol.wt. 35000); 3, myoglobin (mol.wt. 17200); 4, lysozyme (mol.wt. 14300); 5, *Pseudomonas* cytochrome (mol.wt. 9000). ●, Leghaemoglobin.

Fig. 2. *Relationship between molecular weight and retardation coefficient for the same proteins as in Fig. 1*
For details see the text and the legend to Fig. 1.

system revealed that smaller polypeptides were not present and ensured complete denaturation by urea. Kidney-bean and soya-bean leghaemoglobins were indistinguishable in migration, and the apparent molecular weight of each was independent of the gel concentration over the range 5–15%.

The relative mobility (R_F) of a protein at several gel concentrations (T) is described by the equation (Neville, 1971; Rodbard & Chrambach, 1971; Banker & Cotman, 1972):

$$\log R_F = -K_R T + \log Y_0$$

where K_R is the retardation coefficient and Y_0 the limiting relative mobility. The plot of $\log R_F$ against T (Fig. 1) reveals the high Y_0 of leghaemoglobin–SDS, which may reflect either decreased frictional forces or increased negative net charge of this complex. Because the amino acid composition of leghaemoglobin does not deviate from that of an average protein (Dayhoff, 1972), an excessive negative net charge arises only if more than the usual 1.4g of SDS/g of protein is bound. However, leghaemoglobin was found to bind 1.38g of SDS/g and the control, sperm-whale myoglobin, 1.42g/g. Leghaemoglobin thus binds the detergent on the same weight basis as the standard proteins.
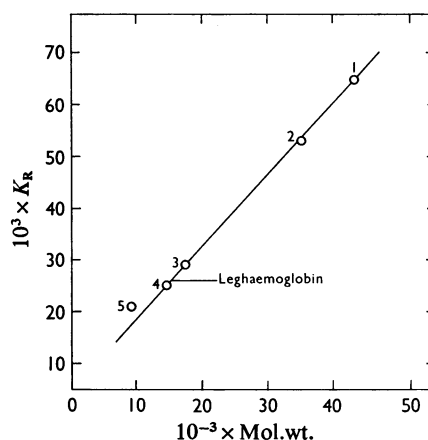
The retardation coefficient K_R is dependent on the effective molecular surface area and, because different protein–SDS complexes ideally lack conformational specificity, on the size of the molecule. An unusual molecular surface area, in particular an unusual conformation of the protein–SDS complex, should be revealed by a K_R value that lies off the line set by the standard proteins in Fig. 2. Leghaemoglobin is normal in this plot and a good molecular-weight estimate of 15200 was obtained.

Because the charge of the leghaemoglobin–SDS complex is normal, the frictional coefficient must be smaller than the molecular weight would imply. On the basis of the retardation coefficients the conformation of leghaemoglobin–SDS is hydrodynamically analogous with those of the standard proteins. Additional evidence of a normal conformation of leghaemoglobin–SDS was obtained from the circular-dichroic spectra, showing that the secondary structures of leghaemoglobin and myoglobin undergo a similar change when exposed to SDS (Table 1). The conformation change occurs immediately with the binding of the first 50% of the detergent.

None of the reasons known for abnormal migration during SDS/polyacrylamide-gel electrophoresis (e.g. poor SDS binding of acidic proteins or proteins containing a rigid S–S bond or carbohydrate, neutralization of the negative charge of SDS by basic proteins, abnormal conformation due to low helical content) is valid for leghaemoglobin. Preferential binding of SDS to the hydrophobic part near the amino end of the leghaemoglobin molecule might possibly lead to electric orientation and thereby decrease the frictional coefficient. The present paper

Table 1. *Effect of SDS on the mean residue ellipticity at 222 nm ($[\theta]_{222}$) and the helical content of sperm-whale myoglobin and kidney-bean and soya-bean leghaemoglobins*

The circular-dichroic spectra were recorded in 50 mm-sodium phosphate buffer, pH 7.0, and the helical contents were calculated as described by Chen *et al.* (1972).

Sample	SDS concentration (mM)	$[\theta]_{222}$ (degree·cm^2·dmol^{-1})	Helical content (%)
Sperm-whale	0	24440	73
myoglobin	4	17440	50
	40	17060	49
Kidney-bean leg-	0	23910	71
haemoglobin	4	17800	51
	40	17130	49
Soya-bean leg-	0	24170	72
haemoglobin	4	18340	53
	40	17820	51

emphasizes that the SDS method is not reliable for all globins, even though members of this group are common standards.

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