ANALYTICAL BIOCHEMISTRY 174, 693–697 (1988)

Stokes Radius Determination of Radioiodinated Polypeptide Hormones by Gel Filtration[1]

MARIA TERESA C. P. RIBELA AND PAOLO BARTOLINI

Division of Medicine, Department of Application of Nuclear Techniques in Biological Sciences, National Nuclear Energy Commission, (IPEN-CNEN) Cidade Universitaria, São Paulo, Brazil

Received April 8, 1988

A simple technique for determination of the molecular (Stokes) radius of radioiodinated proteins was developed using the same column and chromatographic conditions employed in routine radioimmunoassay tracer purification. The calibration curve for five radioiodinated standard proteins presented a highly significant correlation ($r = -0.996$; $P < 0.001$) and allowed precise molecular radius determination for labeled human growth hormone (hGH), luteotropin (hLH), follicle-stimulating hormone (hFSH), thyrotropin (hTSH), prolactin (hPRL), and corticotropin (hACTH), enabling detection of differences of the order of ±3%. The validity of the method was verified by determining the molecular radius of hGH in both "cold" (unlabeled standards and unknowns) and "hot" (radioiodinated standards and unknowns) systems. The technique can be applied in a very simple manner, requiring just one simple additional calibration run before Sephadex G-100 tracer purification. Furthermore, it can be applied to any protein, even when only extremely limited amounts are available. Since the standards and unknowns are labeled and chromatographed under identical conditions, potential common alterations of the molecule due to oxidation, iodine incorporation, tracer–carrier interactions, etc., are automatically corrected for.

KEY WORDS: gel filtration; stokes radius; ^{125}I labeling; anterior pituitary hormones; radioimmunoassay; proteins.

An alteration in the molecular or Stokes radius (1) of a protein, which is directly related to its tertiary structure, may reflect alterations in the biological or immunological properties of the molecule itself (2,3). Moreover, knowledge of the molecular radius of a protein permits more accurate molecular weight determination via alternative hydrodynamic measurements (4–6), as well as being an important parameter in studies of hormone–receptor interactions (7) and molecular self-association (8).

A precise and sensitive technique for Stokes radius determination via gel filtration chromatography of unlabeled proteins has already been utilized in this laboratory (9) to demonstrate that the labeling reagents and iodination per se do not alter this parameter in the case of the hGH[2] molecule. This determination, which can be considered to be quite straightforward for any protein normally available in milligram amounts, can hardly be carried out with good precision in the case of purified hormonal preparations, often available in only extremely limited quantities.

Our approach to this problem was the development of a technique using radioiodinated proteins (both standards and unknowns)

[1] This investigation was supported by CNEN (Brazil), FINEP (Brazil), and IAEA (Vienna, Austria).

[2] Abbreviations used: hGH, human growth hormone; hLH, human luteotropin; hTSH, human thyrotropin; hFSH, human follicle-stimulating hormone; hPRL, human prolactin; hACTH, human corticotropin; hCT, human calcitonin; BSA, bovine serum albumin; OVAL, ovalbumin; STI, soybean trypsin inhibitor; MB, myoglobin from sperm whale skeletal muscle; CYT, horse heart cytochrome *c*.

0003-2697/88 $3.00

and typical chromatographic conditions for routine tracer purification for radioimmunoassay use. As far as we know, the only literature data referring to an analogous method are those reported by Ryan (4), who determined the molecular radii of ^{131}I-labeled hGH, hLH, hTSH, and hFSH. In that work, however, the column purification was carried out under conditions that are not typical of tracer purification and unlabeled standard proteins were employed to define the curve, with only two radioiodinated proteins (bovine serum albumin and chymotrypsinogen) as labeled controls.

For these reasons, in the present work a complete calibration curve was obtained by replicate runs of five ^{125}I-labeled standard proteins, under typical tracer purification conditions (including the presence of a protein carrier); the precision of the method was checked and it was applied to the determination of the six anterior pituitary hormones, which are normally determined by radioimmunoassay. To check the accuracy of the method, the molecular radius of a hormonal preparation easily available in larger quantities (hGH) was also determined in the conventional unlabeled system.

MATERIALS AND METHODS

Purified radioimmunoassay-grade bovine serum albumin (BSA), ovoalbumin (OVAL), soybean trypsin inhibitor (STI), myoglobin from sperm whale skeletal muscle (MB), and cytochrome *c* from horse heart (CYT), as well as highly purified lactoperoxidase, were purchased from Sigma Chemical Company (St. Louis, MO). Human growth hormone was extracted and purified in this laboratory as previously described (10). hLH, hTSH, hFSH, and hPRL were kindly provided by the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK) and National Hormone and Pituitary Program (NHPP) (Baltimore, MD), while human synthetic calcitonin (hCT) was donated by CIBA (Basel, Switzerland). ^{125}I-labeled hACTH was obtained from a commercial kit from Immunonuclear (Stillwater, MN). Sephadex G-100 and Blue Dextran 2000 were products of Pharmacia (Uppsala, Sweden). Na^{125}I, free of carriers and reductants, was purchased from New England Nuclear–Dupont (Boston, MA), at a specific activity of 300–400 μCi/μl.

Radioiodination. The ^{125}I labeling of proteins was carried out via a described modification (9) of the original chloramine-T technique of Greenwood *et al.* (11). In the case of hPRL radiodination the lactoperoxidase method of Thorell *et al.* (12) was used. Standard proteins were labeled at low specific activities, using approximately 150 μCi of ^{125}I for 5 μg of protein; for polypeptide hormones the amount of radioisotope generally employed in routine radioiodinations (~0.7–1 mCi) was used for the same amount of protein.

Stokes radius determination. The general procedure already described in previous work (9) was followed. The chromatographic conditions and buffer composition are detailed in the legend to Fig. 1. The distribution coefficient (K_d) and frictional Stokes radius (R_e) were calculated as described by Martenson (13). The same Stokes radius values for the standard proteins were also used, i.e., 34.8, 28.1, 22.6, 19.0, and 16.5 Å for BSA, OVAL, STI, MB, and CYT, respectively. In the case of human synthetic calcitonin (hCT) the value of 10.0 Å was calculated according to the assumption of Rodbard (14), considering its molecular weight of 3421. In most cases, ^{125}I labeling and running of BSA were unnecessary since it has been demonstrated that this proteins carries ^{125}I during a regular tracer purification (15,16). The precision of the radius determination was calculated via Student's *t* test, as previously reported (9).

RESULTS

Figure 1A presents the calibration curve for Stokes radius determination obtained with unlabeled proteins ("cold" system). Its parameters and degree of reproducibility can

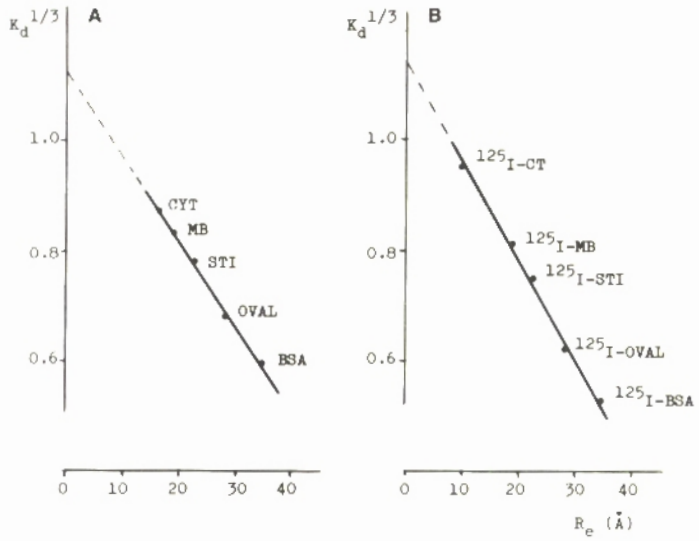

FIG. 1. (A) "Cold" system: calibration curve obtained with a 1.3 × 85 cm. Sephadex G-100 column in 0.05 M phosphate buffer, pH 7.4. Flow rate: 6 ml/h; fraction volume: 1.2 ml. Equation: $K_d^{1/3} = 1.125 - 0.01539R_e$, $r = -0.998$, $P < 0.001$. (B) "Hot" system: calibration curve obtained with a 2.4 × 90 cm. Sephadex G-100 column in 0.05 M phosphate buffer, pH 7.4, 0.1% BSA, and 0.02% NaN_3. Flow rate: 12 ml/h; fraction volume: 2.0 ml. Equation: $K_d^{1/3} = 1.136 - 0.01750R_e$, $r = -0.996$, $P < 0.001$. Each point is the mean of triplicate determinations.

be evaluated in comparison with the different calibration carried out in previous work (9). Figure 1B presents the analogous calibration curve obtained with five radioiodinated standard proteins ("hot" system). As can be seen from the two equations, the correlations were highly significant ($P < 0.001$) in both cases, while a difference of about 14% was observed in the slopes of the two curves ($P < 0.01$).

The sensitivity of the present method for determining small variations in molecular radius, was calculated in the central part of the curve, using ^{125}I-labeled hGH, ^{125}I-labeled MB, and ^{125}I-labeled OVAL: a difference of ±3% in molecular radius can be detected, through triplicate determinations, at a level of significance of $P = 0.05$.

In Table 1, the Stokes radii determined for six different radioiodinated polypeptide hormones are presented together with the standard deviations of n determinations. As we can observe, the interassay coefficient of variation was always less than 3.5%. The radius of hGH was also determined in the "cold" system. A t test showed that the values obtained for hGH in the two systems were not significantly different ($P > 0.1$). Unexpectedly, a significant difference ($P < 0.001$) in hGH molecular radius was observed when this hormone was purified in 0.025 M Tris–HCl, pH 7.4, instead of the routinely used 0.05 M phosphate buffer, pH 7.4. Since the former buffer is often used, especially in radioreceptor assay procedures (17,18), it was deemed interesting to present this divergent value as well. The same table also includes other available literature values for the Stokes radii of these pituitary hormones, together with the corresponding reference, to allow interlaboratory comparison.

DISCUSSION

The technique described here for Stokes radius determination, based on radioiodinated standards and unknowns, has proved to be as accurate and precise as that based on unlabeled proteins, allowing easy detection of

TABLE 1

STOKES RADII OF HUMAN PITUITARY HORMONES

Hormone	Number of determinations	Stokes radius (Å)	C.V. (%)	Literature data	
				Radius (Å)	Reference
^{125}I-hGH	3	22.0 ± 0.26[a]	1.2	22.2	(4)
hGH(phosphate)	9	22.2 ± 0.29	1.3	22.2	(9)
hGH(Tris)	6	26.9 ± 0.50	1.9	24.0	(7)
				18.2–25.6	(19)
^{125}I-hLH	3	27.7 ± 0.50	1.8	30.2	(4)
				30.4	(6)
^{125}I-hLH subunit	2	21.2 ± 0.60	2.8	22.6	(4)
				21.6	(6)
^{125}I-hTSH	3	26.8 ± 0.43	1.6	27.5	(4)
^{125}I-hFSH	3	29.9 ± 0.32	1.1	32.2	(5)
^{125}I-hPRL	3	20.8 ± 0.67	3.2	—	
^{125}I-hACTH	3	11.5 ± 0.30	2.6	—	

[a] Mean ± SD.

scarce hormonal material at the nanogram level. The utilization of ^{125}I-labeled hCT has shown that the calibration curve can be extended down to R_e values of the order of 10 Å, still maintaining an acceptable linearity.

Unlike the technique described by Ryan (4), this determination can be carried out with very little additional work during gel chromatographic tracer purification under the same buffer conditions and in the presence of the routinely used carrier protein. We consider this to be extremely important, having observed remarkable alterations of radioiodinated hormones during chromatography in an unprotected medium (15). The use of radioiodinated standard proteins, possibly presenting the same alterations as the unknowns, should also correct the overestimation error possibly due to increased bond length (C–I versus C–H), mentioned by Ryan (4) as a potential disadvantage of his technique.

The Stokes radii of six radioiodinated pituitary hormones were determined, and comparison with literature data indicates good agreement in the case of hGH and hTSH, while slightly different values were observed for hLH and hFSH. All of them, however, presented a difference always less than 10%. We could not find literature data for hPRL and hACTH.

The agreement with the R_e values for hGH determined by us in the cold system was extremely good, indicating that the difference in slope observed for the two calibration curves does not affect the R_e calculation. Such a difference, which is also observed in Martenson's data, might be due to the different compositions of the elution buffers.

Finally, it should be noted that the Stokes radius of unlabeled hGH is significantly influenced by the ionic strength of the medium, confirming the data reported by Martenson (13) for other, nonhormonal proteins. We observed, moreover, from preliminary results, a similar behavior in radioiodinated hTSH (unpresented data). Considering the possible influence of such an alteration on immunological and biological activity and interactions, this fact should be investigated further. The described technique can therefore also be used as a rapid and inexpensive tool for testing physicochemical homogeneity or identity of trace amounts of radioiodinated proteins.

In our case its application and testing were carried out concomitantly with the labeling and tracer purification, but minor technical modifications (removal of excess ^{125}I, use of smaller samples, different column size, etc.) can allow the handling of much less radioactivity (order of nanocuries) and attainment of higher accuracy. In particular, extending it to the use of HPLC size-exclusion chromatography, we expect to obtain, in much shorter elution times, higher peak resolution with consequent improvement in precision and sensitivity.

ACKNOWLEDGMENTS

We thank Miss Rosangela Arkaten for the excellent technical assistance and Dr. Irene Schwarz for the statistical analysis.

REFERENCES

1. Robinson, R. A., and Stokes, R. H. (1955) Electrolyte Solutions, pp. 1–71, Academic Press, New York.
2. Habeeb, A. F. S. A. (1967) *Arch. Biochem. Biophys.* **121,** 652–664.
3. Jacobsen, C., Funding, L., Møller, N. P. H., and Steensgaard, J. (1972) *Eur. J. Biochem.* **30,** 392–402.
4. Ryan, R. J. (1969) *Biochemistry* **8,** 495–501.
5. Ryan, R. J., Jiang, N. S., and Hanlon, S. (1971) *Biochemistry* **10,** 1321–1330.
6. Bishop, W. H., and Ryan, R. J. (1973) *Biochemistry* **12,** 3076–3084.
7. Roguin, L. P., Bonifacino, J. S., and Paladini, A. C. (1982) *Biochim. Biophys. Acta* **715,** 222–229.
8. Fernandez, H. N., and Delfino, J. M. (1983) *Biochem. J.* **209,** 107–115.
9. Bartolini, P., and Ribela, M. T. C. P. (1986) *J. Immunoassay* **7,** 129–138.
10. Bartolini, P., Assis, L. M., Schwarz, I., and Pieroni, R. R. (1977) *Clin. Chim. Acta* **79,** 223–236.
11. Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963) *Biochem. J.* **89,** 114–123.
12. Thorell, J. I., and Johansson, B. J. (1971) *Biochim. Biophys. Acta* **251,** 363–369.
13. Martenson, R. E. (1978) *J. Biol. Chem.* **253,** 8887–8893.
14. Rodbard, D., and Chrambach, A. (1971) *Anal. Biochem.* **40,** 95–134.
15. Bartolini, P., Assis, L. M., and Fonseca, M. L. Q. (1981) *Clin. Chim. Acta* **110,** 177–185.
16. Bartolini, P., and Camillo, M. A. P. (1983) *Clin. Chim. Acta* **129,** 353–358.
17. Waters, M. J., and Friesen, H. G. (1979) *J. Biol. Chem.* **254,** 6815–6825.
18. Cadman, H. F., and Wallis, M. (1981) *Biochem. J.* **198,** 605–614.
19. Skyler, J. S., Baumann, G., and Chrambach, A. (1977) *Acta Endocrinol.* **75**(suppl.), 5–40.