Vol. 150, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1988 Pages 483-490

PURIFICATION AND CHARACTERIZATION OF PLACENTAL PROTEIN 5

Ralf Bützow[1], Marja-Liisa Huhtala[2], Hans Bohn[3], Ismo Virtanen[4], and Markku Seppälä[1]

1) First Department of Obstetrics and Gynecology, Helsinki University Central Hospital, Helsinki, Finland, 2) Labsystems Research Laboratories, Helsinki, Finland, 3) Behringwerke AG, Marburg, F.R. Germany, 4) Department of Pathology, University of Helsinki, Helsinki, Finland

Received November 30, 1987

This report describes the purification of placental protein 5, PP5, from the human placenta by two affinity chromatography steps, the first with Heparin-Sepharose and the second with Sepharose-linked monoclonal anti-PP5 antibody. The final purification is achieved by reversed-phase high performance liquid chromatography. In SDS-polyacrylamide gel electrophoresis under reducing or nonreducing conditions, PP5 purified in this study migrates as one major band at 36 kD. The previously purified PP5 is more heterogeneous: under nonreducing conditions it migrates at 30 kD and, after reduction, it gives three bands at 16.8 kD, 18.3 kD, and 19.0 kD. In Western blot analysis, both purified proteins react with polyclonal and monoclonal anti-PP5 antibodies. Three N-terminal amino acid sequences are obtained for the previously purified PP5, whereas the N-terminal of PP5 purified in this study is blocked. These results suggest that PP5 previously purified in the absence of protease inhibitors, does not represent the native form of PP5. Computer comparison of the obtained amino acid sequences revealed no significant homology to known protein sequences.

Placental protein 5 is a glycoprotein originally isolated from the human placenta (1). The ability of PP5 to bind heparin (2) and to inhibit plasmin (1,3) and thrombin (4), and our recent finding that PP5 is produced by endothelial cells (5), has linked the action of PP5 to the blood coagulation system. Recent studies have demonstrated PP5 in various tissues and body fluids in nonpregnant women and in men under physiological (6-8) and pathological conditions (9). This suggests that the biological action of PP5 is not restricted to pregnancy.

We purified PP5 from the human placenta in order to carry out its physicochemical characterization, and to compare it with the previously purified PP5 preparation and with other known protease inhibitors and proteins by N-terminal amino acid sequence analysis.

MATERIAL AND METHODS

Preparation of tissue extract. Human term placenta was used as the starting material. Immediately after delivery the placenta was homogenized in 0.015 M sodium phosphate buffer containing 0.9 % sodium chloride (phosphate-buffered saline, PBS), 3 mM ethylenediamine tetra acetic acid (EDTA, Sigma Chemical Corporation, St. Louis, MO, USA), and 3 mM benzamidine (Sigma), pH 7.4. All steps of purification were carried out at 4 °C. After centrifugation for 2 h at

0006-291X/88 $1.50

13,000 rpm, the supernatant was filtered through a 5 cm layer of Sephadex G-50 (Pharmacia, Uppsala, Sweden) on a sintered glass (Table 1).

Heparin-Sepharose affinity chromatography. Heparin-Sepharose (Pharmacia) column (2.2 cm x 4 cm) was used for the first affinity chromatography step (Fig. 1).

Monoclonal anti-PP5-IgG-Sepharose affinity chromatography. Monoclonal mouse anti-PP5 IgG_1-antibodies (clone 56CA5)(5) were purified from mouse ascitic fluid by a protein-A-agarose-based Affi-Gel Protein A MAPS II (monoclonal antibody purification system) kit (Bio-Rad, Richmond, CA, USA) and coupled to cyanogen bromide-activated Sepharose 4 B (Pharmacia) as described (10) (Fig. 2).

Reversed-phase HPLC. Final purification was carried out by reversed-phase HPLC on a C_{18} column (218 TP 54; Vydac, Hesperia, CA, USA) using a Varian 5000 liquid chromatograph, UV-100 detector, and Shimadzu C-R3A integrator. The previously isolated PP5 preparation (1) was further purified by HPLC in the same way before characterization.

SDS-polyacrylamide gel electrophoresis and Western blot analysis. SDS-PAGE was performed according to Laemmli (11) under nonreducing or reducing conditions. After SDS-PAGE separation, the proteins were transferred onto nitrocellulose paper (Trans-Blot®, Bio-Rad) and immunostained using monoclonal anti-PP5 antibody (5)(clone 56CE3; 1:10 dilution of hybridoma culture medium) or polyclonal anti-PP5 antiserum (12)(Lot. 6060A, Behringwerke AG, Marburg, FRG; 1:200 dilution) as described by Towbin (13). Bound antibodies were detected by peroxidase-coupled rabbit anti-mouse or sheep anti-rabbit antiserum (Dakopatts, Glostrup, Denmark) diluted at a ratio of 1:200. The peroxidase reaction was developed by 3,3'-diamino-benzidine-tetra-hydrochloride (Fluka AG, Buchs, Switzerland).

Lectin blotting. After SDS-PAGE, the two purified PP5 preparations were transblotted onto nitrocellulose paper (5 µg/lane). After overnight incubation in PBS containing 3 % BSA, the sheets were exposed to peroxidase-conjugated wheat germ agglutinin (WGA), peanut agglutinin (PNA), or helix pomatia agglutinin (HPA) (1 µg/ml, all from Sigma). In other experiments, the sheets were treated with Vibrio cholerae neuraminidase (0.1 U/ml at 37 °C for 30 minutes) to remove terminal sialic acid before exposure to lectin conjugates. Concavalin A (ConA) binding was studied by exposing the sheets first to ConA (10 µg/ml) and then to rabbit anti-ConA antibodies (10 µg/ml, both from Serotec, Bankside, UK), followed by peroxidase-coupled swine anti-rabbit antibodies (Dakopatts). The specificity of ConA binding was tested by omitting the anti-ConA antibodies, or by inhibiting the ConA binding with 0.2 M α-methyl mannoside (Sigma).

Amino acid sequence determination. Amino-terminal sequences were determined by automated Edman degradation with a gas-phase sequencer (model 470A, Applied Biosystems Inc., Foster

Table 1. Purification of PP5 from placental tissue

Step	Volume (ml)	Total prot.(mg)	PP5 (µg)	Yield (%)	Purification factor
1. Placental homogenate	160	9440[a]	1050	100	1.0
2. Heparin-Sepharose column	11.2	34.72[b]	546	52	142.9
3. Anti-PP5-IgG Sepharose	5	410[b]	320	30.4	9090

a, Measured by the method of Lowry et al.(18). b, Based on absorbance at 280 nm. Absorbance 1.0 corresponds roughly to 1.0 mg protein/ml.

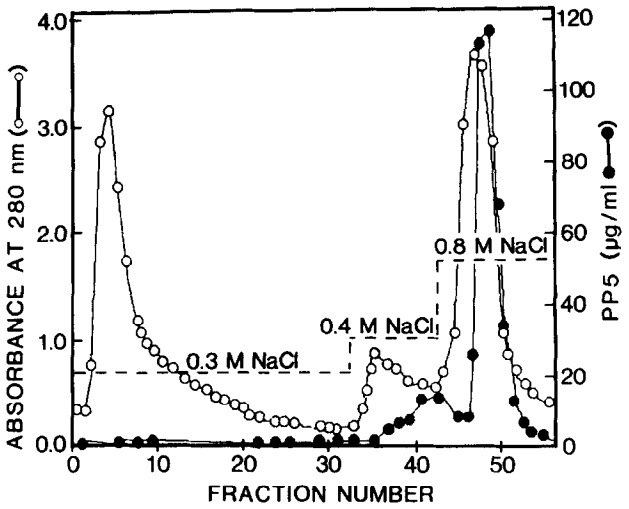

Figure 1. Heparin-Sepharose affinity chromatography of PP5. 160 ml of water-soluble extract from human placenta was passed through a Heparin-Sepharose column pre-equilibrated with 0.015 M NaCl, 20 mM sodium phosphate, 1 mM EDTA, 1 mM benzamidine, and 0.05 % Triton X-100 (BDH Chemicals, Poole, England), pH 7.2. The column was washed with the same buffer and eluted using a stepwise gradient of 0.3 M, 0.4 M and 0.8 M NaCl containing 1 mM EDTA, 1 mM benzamidine and 0.05 % Triton X-100, pH 7.2. PP5 concentration was measured by RIA as described earlier (12).

City, CA, USA). Two approaches were used for determining the N-terminal sequences of the proteins. In the first approach the HPLC-purified proteins (20 µg) were applied directly to a Polybrene/sodium chloride-treated glass fiber filter and degraded in the sequencer (14,15). In the second approach, the HPLC-purified proteins (20 µg) were first run in a 13 % SDS-PAGE, and the separated proteins were transblotted onto a 3-aminopropyl-triethoxysilane-activated fibreglass filter. The blotted proteins were detected by staining with 3,3'-dipentyloxacarbocyanine iodide.

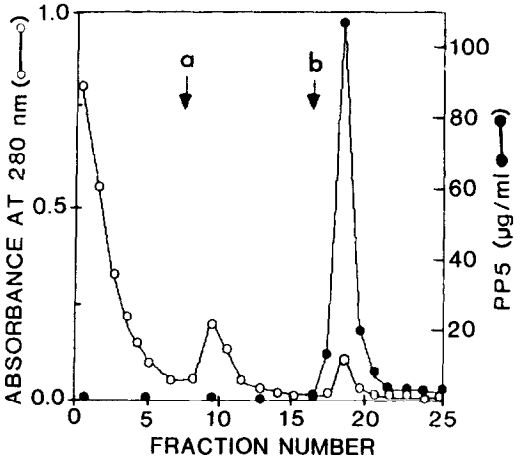

Figure 2. Monoclonal anti-PP5 affinity chromatography of PP5. Major PP5-immunoreactive fractions (number 48-51) from Heparin-Sepharose column were pooled and diluted with 20 mM sodium phosphate buffer with 0.05 % Triton X-100 to give a final NaCl concentration of 0.5 M. The sample was incubated for 24 h with monoclonal anti-PP5-IgG-Sepharose, which then was packed in a column and, after washing, eluted with (a) 20 mM sodium phosphate, 1.0 M NaCl, pH 7.2, and (b) 0.1 M glycine, 6 M guanidine hydrochloride, pH 2.6 adjusted with HCl. Absorbance was measured after desalting with PD-10 column (Pharmacia).

The electroblotted proteins were degraded directly on the activated glass fiber filter after proteins corresponding to the stained protein bands were cut out (16). Amino acid phenylthiohydantoin derivates were analyzed by HPLC on a Spherisorb column (S5 ODS 2, 4.6 x 250) using acetonitrile gradient in the presence of 30 mM sodium acetate, pH 4.8 (17).

Computer comparisons were carried out by the Protein Identification Resource at the National Biomedical Research Foundation (Washington, D.C., USA).

RESULTS

After homogenization and filtration one term placenta yielded 207 ml of extract with a PP5 concentration of 6.5 µg/ml. Thus, the total amount of extracted PP5 was 1345 µg. In reversed-phase HPLC, pooled PP5-immunoreactive fractions from monoclonal anti-PP5-IgG-Sepharose column appeared as one peak eluting at 41 % acetonitrile (Fig.3a).

In SDS-PAGE under nonreducing conditions, the newly purified PP5 appeared as one band at 36 kD (Fig.4, lane 3). In Western blot analysis, polyclonal anti-PP5 antiserum (not shown) and monoclonal anti-PP5 antibody 56CE3 reacted with the nonreduced PP5 at 36 kD (Fig.5, lane 3). Reduced PP5 reacted with polyclonal anti-PP5 antiserum at 36 kD (Fig.5, lane 2). In addition, two faint immunoreactive bands were seen at 20 kD and 21.5 kD (Fig.5, lane 2), which were not detectable by protein staining.

In SDS-PAGE under nonreducing conditions, the previously purified PP5 appeared as two bands: the major band at 30 kD and the minor band at 27.5 kD (Fig.4, lane 4). Upon reduction a single band was observed at 18 kD region (Fig.4, lane 1). In Western blot analysis, polyclonal anti-PP5 antiserum (not shown) and monoclonal anti-PP5 antibody 56CE3 reacted with the nonreduced PP5 at 30 kD and 27.5 kD (Fig.5, lane 4). In addition, bands at 56 kD and 62 kD (Fig.5, lane 4) were seen. After reduction, polyclonal anti-PP5 antiserum reacted with three polypeptides at 19.0 kD, 18.3 kD and, 16.8 kD (Fig.5, lane 1).

In lectin blot experiments, negative results were obtained with the PNA, HPA, and WGA conjugates both before and after treatment with neuraminidase. Both the newly (36 kD) and previously purified PP5 (30 kD and 27.5 kD) reacted with ConA.

HPLC-purified PP5 isolated in this study gave no N-terminal amino acid sequence before or after reduction. By contrast, the previously isolated HPLC-purified PP5 migrating at 30 kD gave three N-terminal amino acid sequences when tested nonreduced. After reduction the same three N-terminal amino acid sequences were recovered from the electroblotted PP5-immunoreactive bands migrating at 19 kD, 18.3 kD, and 16.8 kD (Table 2). Computer comparison of these sequences revealed no significant homology to any previously known protein sequence.

DISCUSSION

The newly purified PP5 appeared as one peak in HPLC and migrated as a single band in SDS-PAGE under nonreducing conditions. The previously isolated PP5 preparation consisted of three

polypeptide chains, as seen in Western blot analysis after reduction. The N-terminal amino acid sequence analysis also revealed three chains. Because no protease inhibition was reportedly used in the previous purification of PP5 (1), the differences between the two PP5 preparations may be due to proteolysis, either *in vivo* or *in vitro*. The heterogeneity observed in Western blot analysis of the newly purified PP5 may also be due to degradation *in vivo* before the addition of the protease

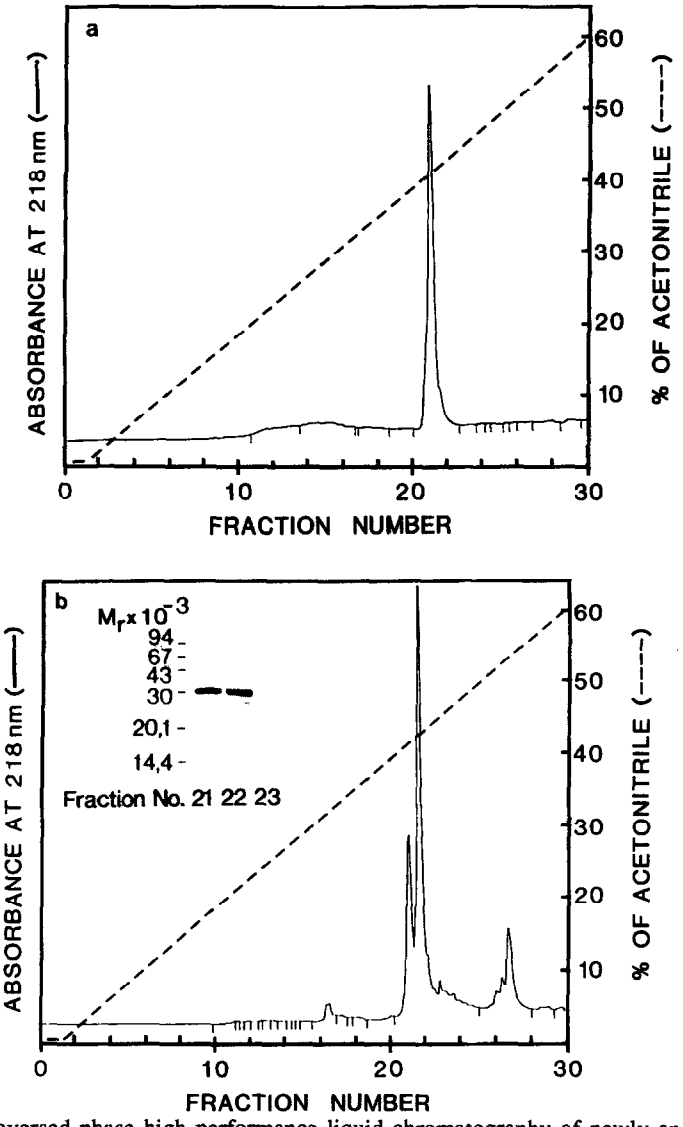

Figure 3. Reversed-phase high performance liquid chromatography of newly and previously purified PP5. (a) 250 μg of PP5 from monoclonal anti-PP5 affinity chromatography was dissolved in 0.1 % trifluoroacetic acid and injected onto a C_{18} column equilibrated with 0.1 % trifluoroacetic acid. A linear gradient of acetonitrile (0-60 %) containing 0.1 % trifluoroacetic acid was used to elute protein. The flow rate was 1 ml/min and 1.0-min fractions were collected and lyophilized. (b) 500 μg of PP5 previously purified was treated in the same way. The fractions were lyophilized and 5 μg of PP5 from fractions 21-23 was analyzed in SDS-PAGE under nonreducing conditions (inset). Fraction 22 was used for further characterization.

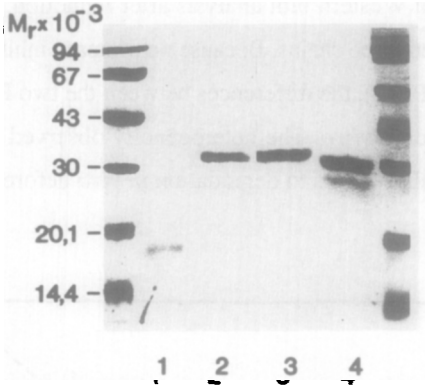

Figure 4. SDS-polyacrylamide gel electrophoresis of PP5. The newly (lanes 2 and 3; 5µg/lane) and the previously purified PP5 (lane 1 and 4; 5µg/lane) were heated for 2 min at 100 °C in SDS-sample buffer without (lanes 3 and 4) or with 2-mercaptoethanol (5 % v/v; lanes 1 and 2), electrophoresed in polyacrylamide gel (15 %) in the presence of SDS, transblotted onto nitrocellulose paper; the proteins were stained with 0.2 % Ponceau S (Chroma, Stuttgart, FRG) with 3 % trichloroacetic acid in H_2O. A low molecular weight electrophoresis calibration kit (Pharmacia) was used as a standard (reduced left; nonreduced right).

inhibitors took place. Results obtained with lectin blotting suggest that PP5 contains N-glycosidic residues, but not O-glycosidic saccharides (19).

PP5 can be distinguished from other serine protease inhibitors on several grounds. Using RIA and polyclonal antibody PP5 is not detected in the serum of nonpregnant individuals (12). This suggests that it is not a major serum protein. Immunologically, PP5 is distinct from the major circulating protease inhibitors such as antithrombin III, α_2-macroglobulin, α_2-antiplasmin, and α_1-proteinase inhibitor, all of which are abundant in normal human serum (20). Two plasminogen

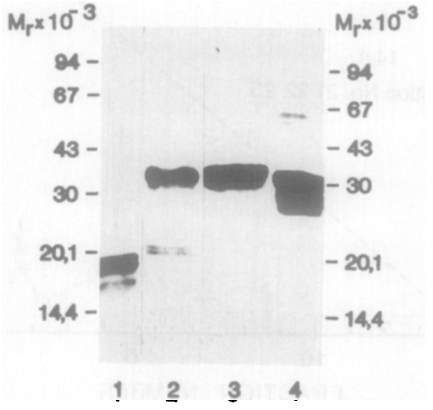

Figure 5. Western blot analysis of PP5. The PP5 transferred to nitrocellulose (Fig.5) was washed with PBS and incubation overnight in 3 % BSA-PBS. After washing, the sheets were cut apart and processed as described in Materials and Methods. Lane 1, reduced original PP5 reacted against polyclonal antiserum; Lane 2, reduced newly purified PP5 reacted against polyclonal antiserum; Lane 3, nonreduced newly purified PP5 reacted with monoclonal antibody (56CE3); Lane 4, nonreduced original PP5 reacted with monoclonal antibody (56CE3). The molecular-size standards are as in Fig.4.

Table 2. N-terminal sequences of electroblotted PP5-immunoreactive proteins

	1				5					10				
19 Kd	Ala	Ala	Ala	Gln	Glu	Pro	Thr	Gly	Asn	Asn	Ala	X	Ile	
18.3 Kd	Asn	X	Ile	Glu	Asn	X	Phe	Pro	X	Glu	Ala	Thr	X	Met
16.8 Kd	Ala	Leu	Leu	Leu	X	Tyr	Tyr							

Positions indicated by X gave no clear assignment suggesting cystein residues at these positions.

activator inhibitors (PAI) have been characterized in pregnancy plasma: the endothelial type (PAI 1) and the placental type (PAI 2) (21). PAI 1 and PAI 2 react with the urokinase-type (u-PA) and tissue-type plasminogen activators (t-PA) only (22), and thus have different substrate specificities from those of PP5. Furthermore, PAI 1 and PAI 2 are immunologically different from PP5 (8). PAI 1 and PAI 2 have been sequenced (23,24), and their structures show no homology to the sequences of PP5. Another plasminogen activator inhibitor, protease nexin I, is produced by a variety of cells *in vitro* (25,26). Like PP5, protease nexin I has binding sites for heparin (27) and it, too, forms complexes with and inhibits several serine proteases. Like in the case of PP5, these proteases include thrombin, trypsin, and plasmin (25). However, the molecular weights of PP5 and protease nexin I differ substantially (36 kD versus 50 kD), and their N-terminal amino acid sequences are different (28)

The previously isolated PP5 may not represent the native form of PP5, but rather a proteolytically cleaved fragment of it. Our results suggest that PP5 is not related to any previously known protease inhibitor.

ACKNOWLEDGMENTS

We thank Dr. U.-H. Stenman and E. Koivunen, M.Sc., for valuable advices. Skillful technical assistance of Ms. Ann Sarpi, Ms. Pipsa Kaipainen, and Ms. Raili Taavela and is gratefully acknowledged. This study was supported by grants from the Emil Aaltonen Foundation (R.B.), the Paulo Foundation (R.B.), the Sigrid Juselius Foundation (I.V., M.S.), the University of Helsinki (I.V., M.S.) and the Foundation for Cancer Research (R.B., I.V.).

REFERENCES

1. Bohn, H., and Winckler, W. (1977) Arch. Gynaekol. 223, 179-186.
2. Salem, H.T., Obiekwe, B.C., Al-Ani, A.T.M., Seppälä, M., and Chard, T. (1980) Clin. Chim. Acta 107, 211-215.
3. Siiteri, J.E., Koistinen, R., Salem, H.T., Bohn, H., and Seppälä, M. (1982) Life Sci. 30, 1885-1891.
4. Meisser, A., Bischof, P., and Bohn, H. (1985) Arch. Gynecol. 236, 197-201.
5. Bützow, R., Virtanen, I., Seppälä, M., Närvänen, O., Stenman, U.-H., Ristimäki, A., and Bohn, H. (1988) J. Lab. Clin. Med., *in press*.
6. Ranta, T., Siiteri, J.E., Koistinen, R., Salem, H.T., Bohn, H., Koskimies, A.I., and Seppälä, M. (1981) J. Clin. Endocrinol. Metab. 53, 1087-1089.

7. Seppälä, M., Tenhunen, A., Koskimies, A.I., Wahlström, T., Koistinen, R., and Stenman, U-H. (1984) Fertil. Steril. 41, 62-65.
8. Bützow, R., Alfthan, H., Julkunen, M., Rutanen, E.-M., Bohn, H., and Seppälä, M. (1986) Hum. Reprod. 1, 287-289.
9. Nisbet, A.D., Bremner, R.D., Horne, C.H.W., and Bohn, H. (1982) Clin. Chim. Acta 119, 21-29.
10. Affinity chromatography. Principles & methods. (1983) Pharmacia, Uppsala, Sweden, pp. 26-27.
11. Laemmli, U.K. (1970) Nature 227, 680-685.
12. Seppälä, M., Wahlström, T., and Bohn, H. (1979) Int. J. Cancer 24, 6-10.
13. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350-4354.
14. Hewick, R.M., Hunkapiller, M.W., Hood, L.E., and Dreyer, W.J., (1981) J. Biol. Chem. 260, 7990-7997.
15. Hunkapiller, M.W., Hewick, R.M., Dreyer, W.J., and Hood, L.E. (1983) Methods Enzymol. 91, 399-413.
16. Aebersold, R.H., Teplow, D.B., Hood, L.E., and Kent, S.B.H. (1986) J. Biol. Chem. 261, 4229-4238.
17. Zimmerman, C.L., Appela, E., and Pisano, J.J. (1977) Anal. Biochem. 77, 569-573.
18. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
19. Lis, H., and Sharon, N. (1986) Annu. Rev. Biochem. 55, 35-67.
20. Laskowski, M.Jr., and Kato, I. (1980) Annu. Rev. Biochem. 49, 593-626.
21. Kruithof, E.K.O., Tran-Thang, C., Gudinchet, A., Hauert, J., Nicoloso, G., Genton, C., Welti, H., and Bachman, F. (1987) Blood 69, 460-466.
22. Sprengers, E.D., and Kluft, C. (1987) Blood 69, 381-387.
23. Belin, D., Vassalli, J.-D., Combépine, C., Godeau, F., Nagamine, Y., Reich, E., Kocher, H.P., and Duvoisin, R.M. (1986) Eur. J. Biochem. 148, 225-232.
24. Ginsburg, D., Zeheb, R., Yang, A.Y., Rafferty, U.M., Andreasen, P.A., Nielsen, L., Dano, K., Lebo, R.V., and Gelehrter, T.D. (1986) J. Clin. Invest. 78, 1673-1680.
25. Scott, R.W., and Baker, J.B. (1983) J. Biol. Chem. 258, 10439-10444.
26. Eaton, D.L., and Baker, J.B. (1983) J. Cell. Physiol. 117, 175-182.
27. Baker, J.B., Low, D.A., Simmer, R.L., and Cunningham, D.D. (1980) Cell 21, 37-45.
28. Scott, R.W., Bergman, B.L., Bajpai, A., Hersh, R.T., Rodriguez, H., Jones, B.N., Barreda, C., Watts, S., and Baker, J.B. (1985) J. Biol. Chem. 260, 7029-7034.