HUMAN HEMOPEXIN. PREPARATION OF THE HEME-RICH PROTEIN

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Received September 18th, 1980

A simplified procedure was developed for the preparation of hemopexin from Cohn fraction IV obtained from partially hemolyzed pooled samples of serum. The method is based on precipitation with rivanol, chromatography on DEAE-cellulose, and gel filtration; it permits large quantities of the material to be treated on a laboratory scale. The preparation of heme-rich hemopexin obtained was characterized by amino acid analysis and the following N-terminal amino acid sequence: Thr-Pro-Leu-Pro-Arg-Gly-Ser-Ala-His-Gly-Asn-Val-Ala-Glu-Gly-Glu-Thr(Thr)Thr--Asn-Pro-Asp-Val-(Gly)(Leu).

Hemopexin is a serum β_1-glycoprotein of molecular weight about 57 000 of which approximately 20% represents the sugar moiety[1]. Hemopexin belongs to specific transport proteins and shows a high affinity for the heme. Its physiological role and physico-chemical characteristics have been described in summarizing reviews[2,3]. The chemical characterization of hemopexin is far from being complete and data on its covalent structure are missing. One of the main obstacles in the way to this characterization has been the lack of an adequate quantity of starting material which, however, has recently decreased due to the advance in the techniques of sequential analysis. Numerous preparative procedures have been recorded in literature which are based on precipitation, ion exchange chromatography, and gel filtration[4–11]. Recently, the isolation of hemopexin has been effected by affinity chromatography based on the affinity of apo-hemopexin for the heme bonded to an affinity support[12–14] or on the interaction with the sugar moiety of hemopexin[15].

This paper describes a modified preparative procedure, related to the work of Hrkal and coworkers[8], permitting relatively large quantities of Cohn fraction IV to be treated on a laboratory scale.

EXPERIMENTAL

Cohn fraction IV was prepared from partially hemolyzed pooled samples in the Institute of Sera and Vaccines, Prague. The material was obtained in the form of a paste containing about 20% of protein in 40% ethanol. The hemopexin content (mostly in heme-hemopexin form) was roughly 2%. DEAE-cellulose Whatman DE 11 or microgranular cellulose DE 32 were products of W. and R. Balston, Ltd., England. Rivanol (2-ethoxy-6,9-diaminoacridine lactate) was purchased from

Zdravotnické zásobování, Prague. The chemicals for automatic sequential degradation were products of Sequanal[R] grade (Pierce, Rockford, Ill., U.S.A.). The remaining chemicals used were of analytical purity.

The composition of the fractions was examined by discontinuous electrophoresis[16] in Tris-glycine buffer, pH 8·3, and by immunoelectrophoresis[17] against horse anti-human serum proteins, anti-hemopexin, anti-albumin, and anti-transferrin serum, products of Sevac, Prague. The determination of hemopexin concentration was effected by radial immunodiffusion[18] on M-Partigen[R] Hemopexin plates using standard human serum; the plates and the serum were products of Behringwerke AG, Marburg-Lahn, F.R.G. The amino acid analyses were performed on 20 and 70 h hydrolysates (6M-HCl, 110°C) by the method of Moore and Stein[19] in an automatic amino acid analyzer[20] (Model 6020, manufactured by the Instrument Development Workshops, Czechoslovak Academy of Sciences, Prague) using the modified procedure of Benson and Patterson[21]. The half-cystine content was determined as cysteic acid after oxidation in performic acid[22]. The N-terminal amino acid sequence of hemopexin was determined by stepwise degradation[23] in Model 890 C Amino Acid Sequenator manufactured by Beckman Instruments, Inc., Spinco Div., Palo Alto, California, U.S.A.) following the "Fast Quadrol Program" recommended by the instrument manufacturer. The degradation products were assayed by thin-layer chromatography[24,25] on silica gel (Silufol sheets, a product of Kavalier, Sázava, Czechoslovakia) and by gas chromatography[26] in Beckman GC-25 chromatograph. The determination of the N-terminal amino acid by the dansyl technique[27] (in the form of 1-dimethylaminonaphthalene-5-sulfonyl derivatives, abb. DNS-) was carried ouf by the modified procedure of Zanetta and coworkers[28]. The chromatography of DNS-amino acids was effected on polyamide layers (Cheng-Chin Polyamide Sheets), purchased from Pierce, in the solvent systems reported by Hartley[29].

RESULTS AND DISCUSSION

The aim of this study was to prepare human hemopexin in a sufficient quantity and a purity complying with the requirements of sequential analysis. Cohn fraction IV, prepared from pooled samples of serum saturated to a considerable degree with the heme and available in larger quantities to us, appeared as one of the possible sources of starting material. In view of the availability of this extensive source the individual isolation steps were chosen so that larger amounts of material could be treated on a laboratory scale.

Since hemopexin is present in the form of the heme complex in the starting material the more recent affinity methods based on supports with the immobilized heme[13,14] cannot be used; the application of another affinity procedure[15] on such a large scale is prevented by the cost of the affinity support. The chromatographic preparative procedure described by Aisen and coworkers[10] used Cohn fraction IV-7 as starting material; this fraction is about ten times richer in hemopexin, predominantly in heme-free form, than our starting material. In view of these facts we decided to isolate hemopexin from blood serum by the procedure of Hrkal and coworkers[8] which was modified and scaled up as described below. The whole preparation was carried out at 4°C.

Precipitation with Rivanol

In a typical experiment 3 kg of Cohn fraction IV in the form of a paste (containing about 600 g of protein) was suspended in 15 l of distilled water in a blendor. The turbid solution, whose pH had been adjusted to 8·0 by 1M-NaOH, was treated with gentle stirring with cooled 1·68% rivanol solution (7·5 l, pH 8·0), added in parts. A bulky, heavy precipitate formed which contained mostly a complex of rivanol and albumin. After standing the yellow-brown supernatant was decanted off. The excess of rivanol was removed from the solution by adsorption to active charcoal added in a quantity sufficient to remove the yellow color. After the charcoal had been removed by filtration through a cotton wool pad and a thick paper filter (Schleicher and Schuell No 589) a solution of red-brown color due to the proteins present was obtained. Unlike in the paper cited[8] excess rivanol was removed from the solution by adsorption to active charcoal[30] (in spite of the fact that an alternate procedure, *i.e.* precipitation with 5% sodium chloride, led to a decrease of the protein losses[31]). This modification of the procedure[8] eliminated the necessity of gel filtration, dialysis, and centrifugation of large volumes of the solutions. Another advantage represented the fact that the ionic strength of the solution did not increase and that it could be directly adsorbed to the DEAE-cellulose column. Because of the efficient chromatographic separation the additional operations, *i.e.* precipitation with ammonium sulfate, gel filtrations, ethanol precipitation, centrifugation, dialysis, and again ultrafiltration, could be deleted. In the final isolation stage we used gel filtration of the hemopexin fraction obtained by ion-exchange chromatography; before gel filtration the volume of the solution was reduced by adsorption to the ion exchanger.

Separation by Chromatographic Procedures

The course of the ion-exchange chromatography of the rivanol supernatant in an informative experiment is shown in Fig. 1. Hemopexin was detected in a double peak E/F showing a marked absorbance at 414 nm. Peak G, also absorbing at 414 nm, contains the residual quantity of albumin after rivanol precipitation; the main component present in peaks C and D is transferrin.

In the preparative experiment (Fig. 2) the load of the column was considerably increased; a part of the contaminating material present in peaks A through C (Fig. 1) was eluted already during the application of the large volume of the samples (the origin in Fig. 2 indicates the start of the gradient elution after the application of the sample). The column was eluted by a gradient of sodium chloride whose steepness was decreased and the separation thus improved. The effluent comprising peaks E and F was treated separately; after dilution with the same volume of distilled water (decrease of ionic strength) and adjustment of pH to 8·0 the solution was applied to a column of DEAE-cellulose Whatman DE 11 (20 × 5 cm), equilibrated with 0·01M-Tris-HCl, pH 7·0. The column was eluted by 0·3M-NaCl in the same buffer;

the main amount of the protein emerged in about 400 ml. After this concentration step, in which the volume of hemopexin-containing fractions decreased more than ten times, the concentrated solution was subjected in parts to gel filtration on Sephadex G-100 (Fig. 3).

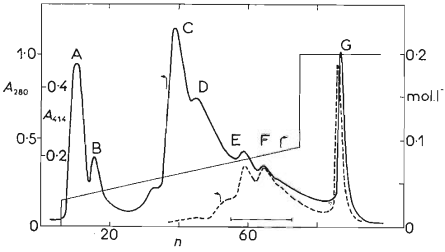

FIG. 1

Ion-exchange chromatography of rivanol supernatant. An aliquot of the solution (pH 7·5), corresponding to 2 g of protein before the precipitation, was placed onto a column (60 × 1·8 cm) of DEAE-cellulose (Whatman DE 37), equilibrated with 0·01M-Tris-HCl (pH 7·0). Elution by a linear concentration gradient of NaCl in the same buffer. Fractions 10 ml/15 min, *n* fraction number; ----- absorbance at 414 nm, ——— absorbance at 280 nm

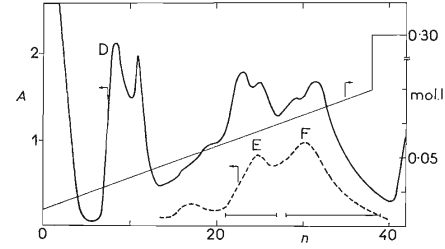

FIG. 2

Ion-exchange chromatography of rivanol supernatant on a preparative scale. The rivanol supernatant (25 l), corresponding to 3 kg of starting material (600 g of protein), pH 7·5, was placed onto a column (60 × 6 cm) of DEAE-cellulose (Whatman DE 11), equilibrated with 0·01M-Tris-HCl (pH 7·0). Elution by linear concentration gradient of NaCl (starting with 35 mM-NaCl) in the same buffer. Fractions 500 ml/30 min. *n* fraction number; ----- absorbance at 414 nm, ——— absorbance at 280 nm

The quantity of starting material treated in repeated experiments was 15 kg (*c.* 3 kg of protein); the yield of fractions E and F was 14·5 and 15·7 g, respectively.

The starting material was compared with the rivanol supernatant and with the preparation obtained by chromatography by discontinuous electrophoresis (Fig. 4). The subsequent assay by immunoelectrophoresis and radial immunodiffusion showed that whereas fraction E contained a considerable admixture of transferrin (not separated from the hemopexin band on discontinuous electrophoresis) and would have required an additional purification[32] for the purpose given, the contamination of the individual preparations of fraction F was 6–10%. The average hemopexin preparation obtained (fraction F) was then characterized chemically.

Chemical Characterization of Fraction F

The amino acid analysis (Table I) of the preparation in which a residual contamination with transferrin was detected was compared with the analytical data on apo-hemopexin obtained by affinity chromatography according to Suttnar and coworkers[14] and with the reported composition of other preparations[15,33]. The comparative

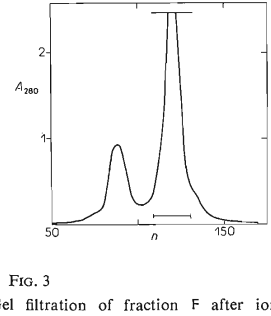

FIG. 3

Gel filtration of fraction F after ion-exchange chromatography. Concentrated fraction F (60 ml) was placed onto a column (80 × 6 cm) of Sephadex G-100 in 0·01M-NH_4HCO_3. Fractions 10 ml/10 min. The material contained in the fractions marked by a bar was treated further.

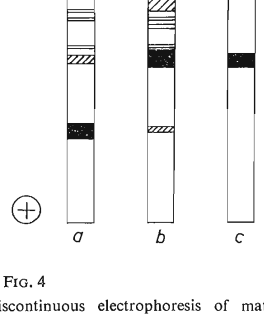

FIG. 4

Discontinuous electrophoresis of material from various isolation steps. *a* Starting material; *b* supernatant after rivanol precipitation; *c* fraction F after ion-exchange chromatography and gel filtration

calculations were based on the assumed content of 34 leucine residues (this number is sufficiently close to the number of 35 leucine residues found by Vretblad and Hjorth[15]). The results given in all columns are comparable in spite of a certain dispersion of the values; an important discrepancy represents the value of methionine (8 residues), obtained in paper[15] whereas the results of other authors point to a value of 5 methionine residues.

Another characteristic of the material, important from the viewpoint of sequential studies, is the determination of the homogeneity of the N-terminal amino acid and of the N-terminal amino acid sequence. The N-terminal amino acid sequence of human and rabbit hemopexin was determined by Morgan and coworkers[34] by stepwise degradation of the proteins. These authors did not identify the products obtained in certain steps of degradation of human material; among others, they were not able to unambiguously identify the N-terminal amino acid. Some data,

TABLE I

Amino acid composition of different hemopexin preparations. The analyses were performed on 20 h hydrolysates, the values were not corrected. The results are expressed as numbers of residues in the molecule of the protein

Amino acid	Fraction F	Apo-hemopexin	Reported analyses	
			ref.[33]	ref.[15]
Lysine	22·4	21·3	21	21
Histidine	17·2	16·8	16	17
Arginine	19·7	19·7	19	21
Cysteic acid[a]	11·5	11·7	10	12
Aspartic acid	38·0	36·5	38	36
Threonine	21·1	20·5	20	21
Serine	25·7	25·8	26	27
Glutamic acid	35·2	33·6	37	36
Proline[a]	30·9	30·1	30	34
Glycine	41·7	40·1	40	38
Alanine	27·0	25·8	27	27
Valine[b]	22·2	23·5	23	22
Methionine	5·2	4·9	5	8
Isoleucine[b]	7·3	8·0	8	9
Leucine	34·0	34·0	34	35
Tyrosine	14·4	13·9	13	14
Phenylalanine	17·5	[c]	18	16
Tryptophan	[c]	[c]	15	13

[a] In oxidized sample; [b] after 70 h hydrolysis; [c] not determined.

however, indicated the presence of a serine residue in position 1 of the hemopexin chain. We degraded automatically in the sequenator fraction F and the preparation of apo-hemopexin[14] obtained by affinity chromatography and compared the data obtained with the results of Morgan and coworkers:

	1	10
Fraction F:	Thr-Pro-Leu-Pro-Arg-Gly-Ser-Ala-His-Gly-Asn-Val-	
apo-hx.:	X-Pro-Leu(Pro)(Arg)Gly(Ser)Ala-His-Gly-Asn-Val-	
ref.[35]:	?-Pro-Phe-Pro-Arg-Gly-Ser-Ala-His-Gly- ? -Val-	
	Leu	

	15	20
Fraction F:	Ala-Glu-Gly-Glu-Thr(Thr)Thr-Asn-Pro-Asp-Val(Gly)(Leu)..	
		(Val)
apo-hx.:	Ala-Glu-Gly-Glu-Thr(Glu)(Thr)Asn-Pro-Asp(Val)(Gly)..	
	(Thr) (Ala)	
	(Val)	
ref.[35]:	Ala-Glu-Gly-Glu-Thr- ? -Thr-Asn-Pro-Asp-Val..	

The amino acid residues given in brackets were not identified unambiguously; the residues shown in brackets below the main line represent contaminants detected in certain cases. The identification of the product obtained in the first degradation step was difficult also in our experiments. The problem was solved by the determination of the N-terminal amino acid of fraction F by the dansyl technique: besides a small contamination with DNS-valine (derived from the admixture of transferrin), DNS-threonine was determined unambiguously, also by chromatography of mixed samples containing DNS-threonine or DNS-serine. The determination of N-terminal threonine confirms the data obtained by Hayem–Lévy and Havez[9]. The finding of phenylalanine and leucine in the third position of pooled material interpret the authors[34] as evidence in favor of genetic polymorphism; only leucine was obtained when the material from a single donor was analyzed. Even though both fraction F and the apo-hemopexin represented pooled material, we observed leucine only in the third degradation step.

The simplified preparative procedure proposed permits considerable quantities of heme-rich hemopexin to be isolated from second-class material on a laboratory scale; the material isolated is suitable for sequential studies.The procedure described was also used for the treatment of normal Cohn fraction IV where the final purification was achieved by affinity chromatography on a support with immobilized heme[32].

We wish to thank the Institute of Sera and Vaccines, Prague, for providing us with the starting material for this study. We thank Mrs J. Rejnková for the carefully performed immunoelectrophoretic assays, Mr J. Zbrožek and Mrs E. Dršková for the amino acid analyses, and Miss J. Chundelová for skilful technical assistance.

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Translated by V. Kostka.