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Isoelectric Focusing and Separation of Hemoglobins of *Myxine glutinosa* L. in a Natural pH Gradient

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Pretreated and untreated hemolysates of *Myxine glutinosa* L. have been subjected to isoelectric focusing and fractionation in a natural pH gradient. Pretreatment, *e.g.* lyophilization of pooled hemolysates, caused formation of many degradation products of hemoglobin including methemoglobin. Untreated hemolysates contained mainly oxyhemoglobin, which could be separated into three major groups; I acidic, isoelectric in the pH region 4.5 to 5.5; II neutral, isoelectric in the pH region 6.5 to 7.1; and III alkaline, isoelectric in the pH region 8.4 to 8.6. Each group contained subcomponents. Two types of hemoglobin patterns could be observed in hemolysates from single hagfish. Possible explanations for these findings are discussed.

In a series of studies on hagfish hemoglobin difficulties were encountered in obtaining a defined and pure material in substantial amounts for chemical analysis. Preliminary experiments indicated that a multiplicity of hemoglobins occur in hagfish blood.[1] To analyse the hemoglobin pattern and to provide pure native hemoglobin, hemolysates of different previous treatment were subjected to isoelectric fractionation according to Svensson[2,3] and Vesterberg and Svensson.[4]

EXPERIMENTAL

Hagfishes from the west coast of Sweden were obtained by courtesy of Dr. B. Swedmark, head of the Kristineberg Zoological Station.

The experiments were performed on material prepared in two different ways.

Hemolysate I. Red blood cells from pooled hagfish blood were collected in a 3.8 % trisodiumcitrate solution in sea water and centrifuged at 16 000 *g* for 10 min and were washed three times with sea-water to remove plasma. The cell sediment was lysed in two volumes of distilled water. Cell debris was removed by centrifugation at 40 000 *g* for 45 min. The supernate was freeze-dried and stored in a vacuum desiccator at 4°C.

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In the next step the freeze-dried material (1 g) was dissolved in 20 ml of 0.05 M phosphate buffer, pH 7.0, and chromatographed with the same buffer on a column (37.5 × 1085 mm) of Bio-Gel P 60 at 4°C, which was equilibrated with the same buffer. The effluent was measured spectrophotometrically at 280 mμ and 410 mμ, the last wavelength being between the Soret-maxima of methemoglobin and oxyhemoglobin.[5] Fractions with a quotient of A_{410}/A_{280} of less than about 3 were not used in further experiments. This value agrees well with the ratio of A_{280}/A_{Soret} of 0.25 given by Lee and Smith [6] for vertebrate hemoglobin, especially if it is taken into account that the absorption peak of the Soret-maximum is very narrow and, therefore, measurements at wavelengths only few mμ beside the maximum will result in a markedly lower ratio of A_{410}/A_{280}.

The fractions containing hemoglobin were eluted in one peak. Concentration of the combined hemoglobin fractions was performed by ultrafiltration with an Ultrafilter LKB 6300 A at 4°C. It was observed that a small amount of material passed the Visking tubing of the ultrafilter, the amount being roughly proportional to the concentration outside the membrane. The concentrated material was freeze-dried again and stored in a vacuum desiccator at 4°C. The resulting substance served as starting material for the first series of experiments on the isoelectric fractionation of Myxine hemoglobin and was subsequently termed Hemolysate I.

Hemolysate II. Blood from one (single hagfish hemolysate) or several (pooled hagfish hemolysate) hagfish, previously narcotized with Sandoz MS 222, lot. No. 55601, 0.05 % (w/v) in sea water, was collected in a 4 % aqueous solution of trisodium citrate by cutting the subcutaneous tail-sinuses (for discussion of the properties of sinus content and venous blood, see Ref. 7). Care was taken to avoid contamination of the samples with slime or other body fluids. The cells were collected by centrifugation at 10 000 g for 15 min. To remove plasma and citrate the blood cells were washed twice with 3.3 % (w/v) of NaCl, and once with a 17 % (w/v) sucrose solution. The cell sediment was lysed by adding one volume of distilled water, and by freezing and thawing. Unlysed cells and cell debris were spun down in a refrigerated centrifuge at 40 000 g for 45 min. This procedure was repeated twice. The supernatants were pooled, and cleared by centrifugation in a Spinco L preparative ultracentrifuge for 45 min at 40 000 g at 4°C. The supernatant from this last centrifugation was decanted and immediately stored at −20°C. Subsequently it was termed Hemolysate II.

The amount of hemoprotein in the samples was calculated from its pyridine-hemochromogen spectrum (Paul *et al.*[8]) using values of molar absorptivity compiled by Falk.[9]

Spectra were recorded with a Cary Model 14 recording spectrophotometer, while single spectrophotometrical measurements were performed with a Zeiss spectrophotometer PMQ II. Reference solution was always water.

The pH measurements were carried out with a pH meter model PHM 25 SE from Radiometer, Copenhagen, equipped with scale expansion, and with a combined glass electrode Type GK 2021 C of the same brand. The glass electrode fitted small test tubes so that measurements could be made on samples smaller than 1 ml. All pH determinations were done at 4°C which was also the temperature of the isoelectric focusing.

The chemicals used in this study were of analytical grade. Carrier ampholytes * were a mixture of low-molecular, aliphatic polyamino-polycarboxylic acids synthesized and fractionated isoelectrically according to Vesterberg.[10] In most experiments carrier ampholytes giving an approximately linear pH course between pH 4 and pH 9 were used. However, for resolving the alkaline hemoglobins a pH gradient essentially between pH 7 and pH 9 was employed.

Preparation and operation of the columns. Three types of columns * were used in the experiments. In early experiments columns of 105 ml capacity were used. These had an external cooling jacket only. Later on, experiments were performed with a new type of column having 110 ml capacity, which was equipped with both an external and a central cooling mantle. With these columns a higher electrical load could be applied, resulting in a shorter focusing time. In two experiments a preparative column * of 500 ml capacity was used.

The columns were loaded according to Ref. 4 using a density gradient of sucrose (from 0 to 50 % (w/v)). The concentration of carrier ampholytes was about 1 % through-

* LKB Produkter AB, Stockholm-Bromma, Sweden.

out the column. The hemolysates were added during the preparation of the density gradient. The central tube surrounding the anode was filled with a 50 % (w/v) sucrose solution containing 0.05 ml orthophosphoric acid. Ethylenediamine (0.05 ml in 3 ml water), was layered above the density gradient where the cathode contacted the solution. In experiments using the preparative column the volumes of acid and amine were increased fivefold. Isoelectric focusing was achieved with a final potential of 800 V (600 V) allowing a maximal effect of 4 watts (2 watts). An excellent focusing was obtained after about 22 h (28 h). The figures in parenthesis refer to experiments run with the older column having an external cooling jacket only.

RESULTS

Hemolysate I. After isoelectric focusing many red and brown zones, and also one green zone, were seen in the column (Fig. 1). The hemoprotein zones were isoelectric in the pH range between 4.5 and 9. Light absorption spectra of the fractions were recorded. Due to the large number of zones with closely

Fig. 1. Isoelectric focusing of Hemolysate I in a column of 500 ml capacity. The anode was at the bottom, the cathode at the top of the column. A pH gradient similar to the one shown in Fig. 3 was used. Only few zones contained mainly oxyhemoglobin. One green zone, probably representing a denatured hemoglobin, was observed in the middle of the column. The rest of the zones visible in the column were brown, thus representing methemoglobin or partially denatured products thereof. The pI's of the main red zones corresponded to those of the main red zones of Hemolysate II Type B. However, most of the other zones found in Hemolysate I could not be found in Hemolysate II, *cf.* Fig. 2.

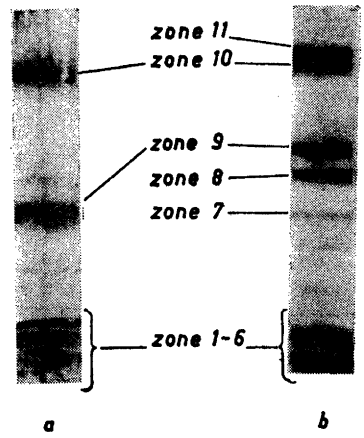

Fig. 2. Isoelectric focusing of Hemolysate II from single fish. The anode was at the bottom, the cathode at the top of the column. The main zones contained oxyhemoglobin. Some of the very faint zones contained methemoglobin.

2a. represents a Type A pattern. For pI values, see Table 1, Expt. No. 6. 2b. represents a Type B pattern. For pI values, see Table 1, Expt. No. 8. Observe the difference between Type A and Type B. The latter having the extra zones 8 and 11. Unfortunately the zones 10 and 11 do not appear well separated in the figure due to the inevitable parallectic error of the camera. They were, however, better resolved in another experiment (see Fig. 5). No special efforts have been made to resolve the acidic hemoglobin zones Number 1 to 6.

spaced pI values it was not possible to separate all the zones in this pH gradient. Furthermore we encountered difficulties in separating neighbouring zones when taking them out of the column. However, it could be shown that the red zones consisted mainly of oxyhemoglobin while the brown zones contained methemoglobin.

Hemolysate II. In order to investigate the cause of the marked heterogeneity of hemoglobin found in Hemolysate I we started experiments on isoelectric focusing of cruder material prepared as Hemolysate II. About one dozen experiments were run on hemolysates from single hagfishes. The yield of hemoglobin from each fish varied considerably but on the average approximately 1.4 μmoles could be obtained in the hemolysate from a single fish, as has been determined by the pyridine hemochromogen method. After isoelectric focusing fewer zones were observed with Hemolysate II than with Hemolysate I. Moreover, almost only oxy-Hb zones were seen in the column. A closer inspection of the isoelectric distribution of zones revealed two distinctly different types of hemoglobin patterns. *Type A:* characterized by a single oxy-Hb zone with pI 7.0 (zone No. 9) in Group II, and a single oxy-Hb zone with pI 8.4 (zone No. 10) in Group III (Fig. 2a). For results of analyses of fractions see Fig. 3. *Type B:* having two oxy-Hb zones in Group II, *i.e.* No. 8 and No. 9 with pI 6.7 and 7.0, respectively, and two oxy-Hb zones in Group III, *i.e.* No. 10 and No. 11 with pI 8.4 and 8.6, respectively (Fig. 2b). For results of analyses of fractions see Fig. 4.

The two main alkaline zones, No. 10 and No. 11, of Type B pattern could be resolved much better by using a more shallow pH-gradient. (Fig. 5). Another main oxy-Hb zone with pI 5.2 was also observed in both patterns. Several more acidic oxy-Hb zones seemed to be variable. Sometimes very faint oxy-Hb zones could be seen with a pI of 5.4 and 6.3. Experiments to separate and study the acidic oxy-Hb zones are under way. Light-brown zones, probably consisting of methemoglobin, could also be seen in the columns. They increased somewhat during the run.

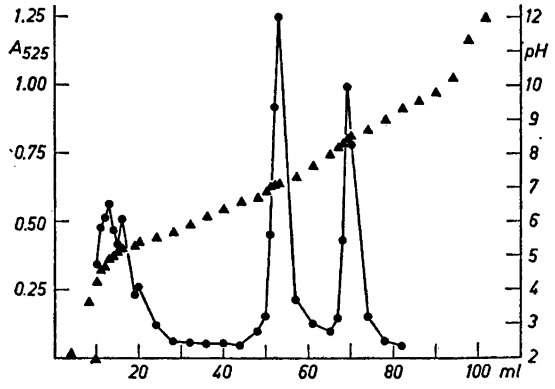

Fig. 3. Results of analyses of fractions from an experiment similar to the one presented in Fig. 2a: ▲, pH and ●, A_{525}. For pI values and the amounts of hemoglobin in the peaks, see Table 1, Expt. No. 6.

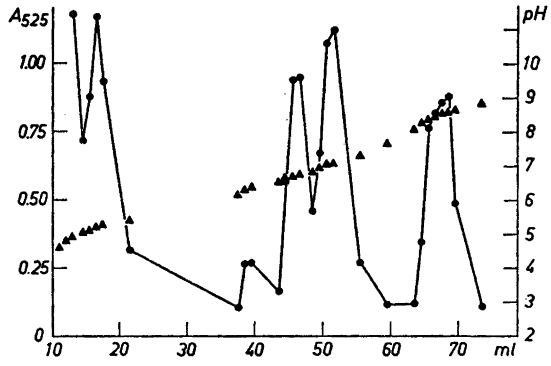

Fig. 4. Results of analyses of fractions from the experiment represented by Fig. 2b: ▲, pH and ●, A_{525}. For pI values and the amounts of hemoglobin in the peaks, see Table 1, Expt. 8.

It was possible to obtain oxy-hemoglobin free of methemoglobin in the zones with alkaline pI, Group III, after isoelectric focusing of Hemolysate II. In Table 1 the pI values and the amounts of oxy-Hb of the main zones are shown. Due to turbidity of the samples of the first four zones the absorbance at 525 mμ is rather high, resulting in artifactually elevated hemoglobin contents of the zones. The millimolar absorptivity at 525 mμ was taken as 9.52. This value was derived from 17 determinations of the pyridine-hemochromogen on hemoglobin samples of which the spectra were recorded. The wavelength of 525 mμ was chosen for the quantitative determination of hemoglobin because this is an isosbestic point of the spectra of oxy- and methemoglobin (*cf.* Horecker in Lemberg & Legge, Ref. 11, p. 299). By this procedure, errors due to conversion of oxy-Hb to met-Hb, could be avoided. It was found that the acidic oxy-Hb had a tendency to transform slowly into met-Hb.

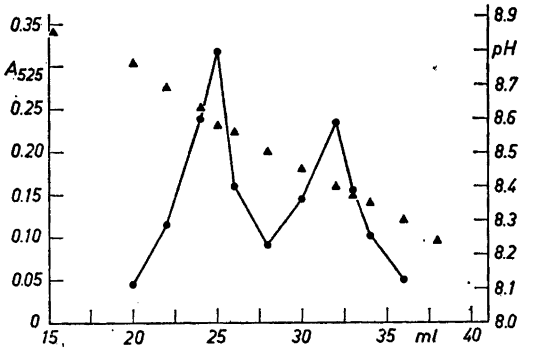

Fig. 5. Results of analyses of fractions from an experiment performed for the separation of the most alkaline hemoglobines zones number 10 and 11, shown in Fig. 2b. ▲, pH and O, A_{525}.

DISCUSSION

When a protein is found to be heterogeneous in a new separation procedure, it is of interest to determine if the separated components are artifactual. Many experiments have been carried out with the method of isoelectric separation of proteins, *e.g.* myoglobin,[4,12] lactoperoxidase,[13] and cytochrome c,[14] which show heterogeneities which have been well characterized by such methods as electrophoresis and ion exchange chromatography. In no case have artefacts been observed, and the method of isoelectric focusing has been shown to possess a very high resolving power for the separation of proteins with charge differences making their separation by other methods difficult.

We have also employed other techniques to check the results of hagfish hemoglobin heterogeneity. Experiments with agar-gel and acrylamide-gel electrophoresis on microscope slides, as developed by Wieme,[15] were carried out with the Vitatron equipment. With the Hemolysates I and II several bands were obtained. Due to the small amount of hemoprotein in a single band on the slides, it was not possible to distinguish between oxy- and methemoglobin. This was one of the limitations to the evaluation of the electrophoresis patterns. To increase the sensitivity of detection of hemoprotein bands, the gel slabs were stained with benzidine and hydrogen peroxide. When running material from Hemolysate I, more than 12 zones showed up after staining, indicating great heterogeneity. Hemolysate II separated into 4 to 6 benzidine-positive zones. This could very well reflect the results obtained by isoelectric focusing. However, the resolution of zones by electrophoresis was much inferior to that achieved by the isoelectric separation method. When material from a component separated by isoelectric focusing was examined by electrophoresis only one band was obtained.

The resolving power of the method of isoelectric focusing depends on the slope of the pH course.[4] The most favourable pH course used in this study for the separation of hagfish hemoglobin was that applied in the experiment shown in Fig. 4, in which the most alkaline hemoglobins have been separated. For this experiment it was calculated that two proteins should separate partially if they differed in pI by 0.08 pH units. It is of course easier to observe closely spaced hemoglobins within the column than by analysis of fractions due to the inevitable tailing of zones during the draining procedure.

We regard the characterization of separated components by their respective pI values as very valuable. For instance, it is possible to identify a certain component in different experiments by its pI vaue. It can be seen from Table 1 that the pI values of the hemoglobins can be determined with a reproducibility of about ± 0.04 pH units. The reproducibility depends among other factors on the value of the pH gradient: the steeper the pH course, the less accurate is the pI determination and the less fine is the resolution and *vice versa*. Detailed studies in shallower pH courses over smaller pH ranges would increase the reproducibility of the pI determinations and at the same time increase the resolving power.

It is not easy to explain the extra zones obtained with Hemolysate I. The total amount of methemoglobin was much greater in Hemolysate I than in Hemolysate II. It cannot be assumed *a priori* that the difference in pI

Table 1. Data on oxyhemoglobin zones separated by isoelectric focusing of Hemolysate II.

Expt. No.	1 [a]	2 [a]	3 [b]	4 [b]	5 [a]	6 [a]	7 [a]	8 [a]
Zone 1 pI nmole						4.55	4.55	4.55
Zone 2 pI nmole					4.80			4.85 458 *
Zone 3 pI nmole			5.00 177	4.95 125	4.95 315 *	4.95 285 *	4.90	
Zone 4 pI nmole							5.10 313 *	
Zone 5 pI nmole	5.15 182	5.15 380	5.25 92		5.20 340	5.15	5.20	5.20 503
Zone 6 pI nmole						5.45 200 **	5.45 313 **	
Zone 7 pI nmole				6.30			6.35 79	6.35 136
Zone 8 pI nmole					6.75 286			6.70 442
Zone 9 pI nmole	7.00 465	6.95 815	7.00 511	7.05 266	7.05 301	7.10 399	7.10 437	7.05 491
Zone 10 pI nmole	8.40 249	8.40 593	8.45 398	8.40 261	8.40	8.45 326	8.45 257	8.54
Zone 11 pI nmole					8.60 342 **			8.60 543 **

[a] quantification of hemoglobin from absorbance at 525 mμ; [b] quantification of hemoglobin from pyridine-hemochromogen spectrum;

* total amount of all previous (more acidic) zones; ** from that and the previous zone.

Note: Values of hemoglobin content of the acidic zones determined by absorbance measurements at 525 mμ are probably too high due to the turbidity of the samples.

between one oxyhemoglobin and its methemoglobin form should have the same value for all oxyhemoglobins observed. Therefore it is difficult to assign the methemoglobin zones to their respective oxyhemoglobin zones. A rough estimate of the difference in pI for an oxy- and the corresponding methemoglobin gives a figure of some tenths of a pH unit, which means that they should be separated in the column. In the isoelectric patterns of Hemolysate II only very faint methemoglobin zones were detectable. In order to give an idea of possible reasons for the artefacts in Hemolysate I, it may be mentioned

that sulphydryl groups have been found in hagfish hemoglobin. Therefore this protein might form mixed disulphides (*cf.* Refs. 16, 17). Moreover, freezing and also drying have been responsible for methemoglobin formation and denaturation of hemoglobin (Ref. 11, p. 396 and Refs. 18—20). Freezing and thawing a mixture of the main components of lactate dehydrogenase (LDH) is reported to give proteins having the same electrophoretic mobility as some of the isoenzymes of LDH.[21] Storage at +4°C decreases enzyme activity of LDH isoenzymes at different rates.[22] On the other hand the effect of freezing and thawing a hemoglobin component isolated by isoelectric fractionation seems to be small. The most alkaline hemoglobins have been stored for several days at −20°C, and after thawing have been submitted to isoelectric focusing for a second time. The resulting pattern was identical to the pattern observed after the first isoelectric focusing experiment (see Fig. 5). Furthermore, the small amount of methemoglobin found in Hemolysate II indicates that the preparation procedure used with this type of hemolysate is very mild. The importance of a method which can be used for purification of macromolecular ampholytes and for revealing artefacts at the same time is evident.

Hemoglobin heterogeneities are often shown by gel-electrophoresis using microquantities. Obviously there is a risk in drawing conclusions from results of separations obtained by a technique using amounts of material too small to permit subsequent analyses of the components by other methods. For example, it is important to be able to analyze whether the components separated are oxy- or methemoglobin. It is, of course, also necessary to try to reveal artefacts created during the preparation procedure.

Several papers have been published on hemoglobin heterogeneity in which different hemoglobin components were separated by electrophoresis and conclusions were drawn on the genetic background.[23,24] Other explanations have also been proposed for electrophoretic heterogeneity of hemoglobins, *e.g.* the sequential induction of synthesis of different globin chains during ontogenetic development,[25,26] and age-dependent functional changes of erythrocytes together with the hemoglobin contained in them.[27,29] References concerning the heterogeneity of hemoglobin in fish and cyclostomes are given in Refs. 30—35. Keeping the above-mentioned possibilities in mind, we are somewhat reluctant to interpret the isoelectric patterns obtained on Hemolysate II.

With the isoelectric separation method it is possible to handle enough material for the characterization of the components by chemical methods, *e.g.* amino acid analysis, "fingerprinting", N- and C-terminal sequence analyses, *etc.* However, we do not yet regard the separated components as adequately purified for such analyses, because the possibility exists that proteins of different composition have nearly identical pI values and overlap within the column more or less. Therefore, experiments with shallower pH courses should be carried out in order to increase the purity of the components. In such experiments hemoglobins with pI values more than one pH unit apart should be run separately. Since the isoelectric focusing depends on the overall charge, or in other words, on the pI value of the molecule, it seems advisable to use in addition a method based on another property of the substance, *e.g.* a separation procedure based on molecular size, for instance molecular sieve chromatography with Sephadex. In this connection it may be mentioned

that carrier ampholytes can be separated from proteins by chromatography on a short column of Sephadex G 15 or G 25 equilibrated with buffered 1 M NaCl. A high ionic strength is used to dissociate ampholyte-protein complexes which may occur.

Experiments with hemoglobins of pI at 5.2, 7.0, and 8.4 indicate that the hemoglobins isolated by isoelectric separation are quite homogeneous in sedimentation experiments in the ultracentrifuge and that the components have roughly the same molecular weight of about 21 000.[36]

In our material Type A and Type B hemoglobin patterns seem to occur in the same proportion. It is not clear if the Types A and B represent genetically different forms or developmental changes comparable to those found in the brook lamprey [25] and in Man,[24] or whether they represent other, perhaps adaptive, alterations (*cf.* Ref. 34).

From the discussion above it is clear that considerable work remains to be done before the heterogeneity of hagfish hemoglobin can be well understood.

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