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A Microscale Isolation of Hemins from Hemeproteins by Use of Polyacrylamide Gel Electrophoresis[1]

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An analytical and semipreparative procedure has been developed for the isolation of the prosthetic groups of various hemeproteins by use of polyacrylamide gel electrophoresis. Electrophoresis of erythrocyte formylhemeprotein in the presence of 20 mM cyanide and subsequent elution of the sharp hemin band resulted in 87% recovery of hemin and isolation of apoprotein. Removal of the protohemin prosthetic groups from methemoglobin, metmyoglobin, horseradish peroxidase and erythrocyte cytochrome b_5 were also effected (in 47–80% yield) if urea as well as cyanide were present. The procedure is straightforward, rapid, gives good yield from only a few nanomoles of protein, and has the important advantage of avoiding the use of acidic conditions and organic solvents employed in conventional heme extraction procedures.

Hemins have been classically isolated from hemeproteins by extraction of an acidic solution of the protein with a nonpolar solvent. The acid conditions dissociate the hemin from the protein presumably by protonation of the ligands and side chains of the hemin. Acid/acetone extraction (1) has been successfully employed for isolating protohemin from a large number of hemeproteins, heme *a* from cytochrome oxidase (2), siroheme from sulfite reductases (3), and chlorocruoroheme from chlorocruorin (4). This procedure has been successfully modified by using different acids and different nonpolar solvents including diethylether, methylethyl ketone, butanol, and ethyl acetate. Hemins have also been isolated by use of strontium chloride hexahydrate in glacial acetic acid (5). The disadvantage of these procedures is that modification of labile prosthetic groups and irreversible denaturation of the resulting apoprotein may result from the acidic conditions and/or the nonpolar solvent.

Procedures for hemin extraction which avoid acidic conditions have therefore been developed. Protoheme has been removed from hemoglobin by continuous extraction with methanol (6) and by extraction with acetone in the presence of nitric oxide or imidazole derivatives (7). The prosthetic groups of cytochrome *b* and cytochrome oxidase have been extracted with pyridine in the presence of deoxycholate (8). Heme *a* has also been extracted with chloroform/pyridine (9).

Attempts to isolate the formylheme prosthetic group of the erythrocyte green hemeprotein (10) by acid/acetone have been fruitless, while ethyl acetate/acetic acid (4:1) extractions have yielded spurious results and poor yields (11). The application of membrane filtration of the protein in the presence of pyridine/alkali (12) was an improvement over the ethyl acetate/acetic acid procedures as judged by various criteria such as paper chromatography and visible spectra. Overall yields however were still quite low and the procedure was quite time-consuming.

This communication describes a gentle and rapid electrophoretic method for the isolation of hemins from very small quantities of hemeproteins. It is a simple adapta-

[1] Preliminary accounts of these studies have been presented at the 59th Annual Meeting of the Federation of American Societies for Experimental Biology, April 13–18, 1975, Atlantic City.

tion of conventional discontinuous polyacrylamide-gel electrophoresis. Partial dissociation of the hemin from protein by cyanide or by urea plus cyanide and simultaneous electrophoresis successfully allows isolation in unaltered form of even the very labile prosthetic group of the erythrocyte green formylhemeprotein.

MATERIALS

Tris base, L-histidine, and ferriprotoporyphyrin 9 were obtained from Sigma; glycine and sucrose from Matheson, Coleman and Bell; *N,N,N',N'*-tetramethylethylenediamine and Photo-flo 200 from Eastman; acrylamide and bisacrylamide, both electrophoresis purity grade, and Bio-Gel P-4 from Bio-Rad; bromophenol blue from Allied Chemical; bovine methemoglobin from Pentex; whale skeletal muscle metmyoglobin from Calbiochem; and horseradish peroxidase from Worthington. Red blood cells from recently outdated human blood were a gift from the University of Michigan Medical Center Blood Bank. Urea, obtained from Fisher, was recrystallized from hot 70% ethanol containing 1 mM EDTA. Distilled and deionized water was used throughout. Soluble cytochrome b_5[2] and formylhemeprotein[3] were isolated in highly purified form from bovine erythrocytes. Purified rabbit liver cytochrome P-450 was a gift from Dr. Minor Coon.

METHODS

Preparation of hemoglobin and methemoglobin. Human hemoglobin was obtained from 3-week-old units of blood. The cells were washed three times at 4°C by centrifuging a suspension of cells in cold 0.9% NaCl solution at 2000g for 10 min and then aspirating off the buffy coat and supernatant fraction. Cells were lysed by the addition of three volumes of cold water. After the pH was adjusted to 6.0 the stromata were sedimented by centrifugation at 13,000g for 60 min and discarded. Three volumes of cold water were added to the supernatant fluid and the pH was adjusted to 7.2. The diluted supernatant fluid was then passed through a DEAE-cellulose column of one-tenth its volume that had been equilibrated with 3 mM potassium phosphate buffer, pH 7.2. The hemoglobin obtained was used immediately. When conversion of the hemoglobin to methemoglobin was desired, 100 μmol were incubated with 2 mg of potassium ferricyanide for 5 min at room temperature. Salts were subsequently removed by the centrifugal method of Neal and Florini (13), except that Bio-Gel P-4, equilibrated in 3 mM potassium phosphate, pH 7.2, was used instead of Sephadex G-25.

Preparation of the gels. Polyacrylamide gels were prepared and electrophoresis was carried out according to the method of Brewer and Ashworth (14) except that no stacking gel was used. Instead, samples were made 10% in sucrose and were layered under the electrophoresis buffer on top of the gel with a Hamilton syringe. The glass tubes (8 × 0.5 cm) for the electrophoresis were thoroughly cleaned and then rinsed with Photo-flo:water (1:50) in order to facilitate the eventual removal of the gels from the tubes. The gels were usually composed of 7.5% acrylamide, but the acrylamide concentration was increased to 10 or 15% if there was any chance that contaminating proteins were migrating with the hemin. Each gel was formed from 1.2 ml of acrylamide solution.

When electrophoresis was performed in the presence of urea, the bottom two-thirds of the running gel was formed first in the regular way in the absence of urea in order that no urea would be isolated with the hemin. The upper third of the gel was made 6.25 M in urea by including urea in the various solutions used to make the gel. The yield of hemin was the same whether urea was present in the whole gel or only in the top third. Urea must be included in the top part of the gel since release of the hemin from the protein continues as the protein proceeds down the gel. When 6.25 M urea was used in the upper third of the gel only, the hemin band migrated away from the urea and little urea contaminated the isolated hemin.

Preelectrophoresis. For the electrophoresis of formylhemeprotein, the urea-free gels were subjected to a prior electrophoresis in the presence of sodium thioglycollate (14, 15) to remove any residual free radicals and persulfate which might react with the very labile prosthetic group or the apoprotein. Sodium thioglycollate (2 μg) and a small amount of bromophenol blue in the running buffer were applied to each tube and preelectrophoresis was conducted with upper and lower reservoirs containing running buffer diluted to the same concentration as the buffer in the gel. Preelectrophoresis was carried out at 4°C and at 5 mA/tube until the tracking dye had migrated to the bottom of the tubes.

Preparation of the samples. In those runs where urea was used, the sample proteins were dissolved directly in 8 M urea (pH found to be 8) rather than in buffer plus sucrose. The omission of electrophoresis buffer did not affect the yield but did enhance the rate and degree to which hemeprotein concentrated into a sharp band. The samples in urea solution were incubated at 37°C for at least 0.5 h before application to the top of the gel.

Electrophoretic separation of hemin from protein. Electrophoresis of the samples was performed at 4°C at 2 mA/tube for the first 20 min and then at 4–6

[2] In preparation; Douglas, R. H., and Hultquist, D. E.

[3] In preparation; DeFilippi, L. J., and Hultquist, D. E.

mA/tube until the hemin band was within 1 cm of the bottom of the gel. Cyanide was included in the upper electrophoresis reservoir only. If the voltage was found to exceed 200 V/gel after the protein had entered the gel, the urea solution remaining on top of the gel was removed with a Pasteur pipet. After electrophoresis the gels were extruded with water pressure and rinsed in water. Gels were scanned directly at 280 nm for protein and at the Soret peak for hemeprotein and hemin. Scanning was carried out at 1 cm/min in a Gilford spectrophotometer equipped with a linear transport attachment and a #2410 slit plate (0.05 × 2.36 mm). When necessary, protein was also detected by staining with 1% amido black after fixing in water/acetic acid/methanol (88:7:5).

Isolation and quantitation. For isolation of hemins, the gel was frozen on a block of Dry Ice, and the part of the gel containing the hemin band was cut out with a scalpel, chopped into small pieces, and dispersed by pressing through the orifice of a 0.5-ml syringe. The hemin was extracted from the gel by shaking with water or water/methanol (4:1). Yields were calculated from the absorbance of the reduced pyridine hemochromes (16) of the hemeproteins and the isolated hemins. Pyridine hemochrome spectra were recorded with a Cary Model 14 recording spectrophotometer. For formylhemeprotein the absorbance was read at 434 nm. For protohemin-containing proteins the difference in absorbance between the α peak and the trough between the α and β peaks was calculated, and these values used to determine the yield of the hemin.

Isolation of the apoprotein. For isolation of the apoprotein of the erythrocyte formylhemeprotein, the gels were scanned directly at 280 nm to detect the apoprotein and at the Soret peak to distinguish the apoprotein from residual hemeprotein. The gels were dispersed and eluted with 0.1 M sodium phosphate buffer, pH 7.2, containing 0.145 M sodium chloride.

RESULTS

The electrophoretic procedures resulted in a sharp, bright-red band of cyanide–hemin complex that was cleanly separated from the apoprotein and any remaining hemeprotein. Table I shows the yields of hemin for each of the ferrihemeproteins following electrophoresis in the presence of cyanide, urea, and cyanide plus urea. The hemin of the erythrocyte formylhemeprotein was easily displaced from the protein, 20 mM cyanide being sufficient to obtain an 87% yield. In contrast, cyanide alone was not effective in isolating the hemin from the other hemeproteins tested, but, when used in conjunction with 8 M urea in the sample plus 6.25 M urea in the gel, moderate to high yields were obtained.

A prior incubation of the urea-containing samples at 37°C was necessary for a good yield upon subsequent electrophoreis. When human methemoglobin was incubated with 8 M urea at 0°C for 1 h, the yield of hemin was one-fourth the yield obtained after a 37°C incubation. Parallel results were obtained for sperm whale metmyoglobin, the yield dropping to one-third. Inclusion of 20 mM histidinate rather than cyanide in the upper reservoir buffer also resulted in release of hemins but in a much lower yield. Release of protohemin from

TABLE I

YIELD OF HEME FROM HEMEPROTEINS

Hemeprotein	Amount of hemeprotein (nmol heme/gel)	Percent yield		
		Urea[a]	Urea[a] + KCN[b]	KCN
Erythrocyte formylhemeprotein[c]	8.5	—	—	87[d]
Bovine methemoglobin[e]	23.2	26	80	<5[b]
Sperm whale metmyoglobin[e]	23.2	9	47	<5[b]
Horseradish peroxidase[e]	10	23	71	<5[b]
Erythrocyte cytochrome *b*5[f]	6.4	—	53	—
Cytochrome P-450[e]	2.4	—	0	0[b]

[a] 6.25 M in gel, 8 M in sample.
[b] 40 mM in the upper electrophoresis reservoir buffer.
[c] 10% acrylamide gel.
[d] 20 mM in the upper electrophoresis reservoir buffer.
[e] 7.5% acrylamide gel.
[f] 15% acrylamide gel.

cytochrome P-450 did not occur under any of the tested conditions. The intact cytochrome P-450 simply collected on the surface of the gel.

The oxidation state of the heme appeared to be important, the ferric heme being the form that was prone to removal from the protein. This was shown in the following experiment using hemoglobin as a model since its ferrous heme is resistant to oxidation. Human ferrous and ferric hemoglobin were obtained as described in the methods section. Both forms were incubated in 8 M urea at 0°C for 1 h and then electrophoresed in the same fashion as were the other protohemin-containing proteins. The incubation was at 0°C in order to delay oxidation of the ferrous form. Electrophoresis of methemoglobin resulted in a sixfold greater yield of heme plus hemin over ferrous hemoglobin. In a control experiment metmyoglobin gave essentially the same yield as metmyoglobin treated with potassium ferricyanide.

The electrophoretic separation of formylhemeprotein into hemin and apoprotein is illustrated in Fig. 1. Following electrophoresis of the intact protein in the absence of cyanide, subsequent scanning of the gels at 416 nm for hemin and hemeprotein (Tracing A) illustrates that only a small amount of hemin was present as the fast-migrating free hemin and that most was present as a component of the major and minor forms of the intact protein. However, electrophoresis in 30 mM cyanide (Tracing B) resulted in a large amount of free hemin with only small amounts of hemin still bound to the protein. Scanning of the same gel at 280 nm (Tracing C) showed that the apoprotein migrates slightly more slowly than the major form of the hemeprotein and was cleanly resolved from the free hemin (which also absorbs light at this wavelength). A gel similar to this, stained with amido black, showed no protein migrating with the hemin. Apoprotein, located by the 280-nm scan, was eluted and found to give a strong, single precipitin band by Ouchterlony double diffusion when reacted against antibody prepared against the holoprotein, indicating that no apparent denaturation had taken place.

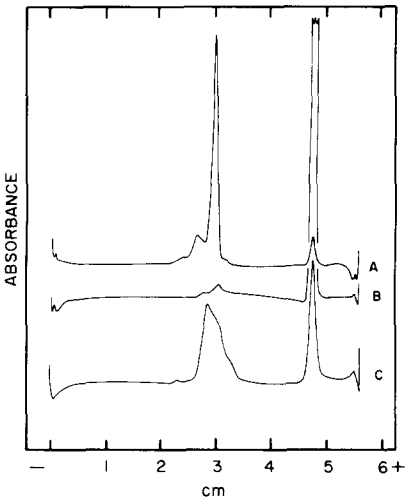

FIG. 1. Electrophoretic separation of the heme and apoprotein of erythrocyte formylhemeprotein. Electrophoresis was performed on a 10% gel and subsequent scanning of gels carried out as described in Methods. The direction of migration toward the anode is from left to right. (A) Electrophoresis of 2.9 nmol of hemeprotein in absence of cyanide. Gel was scanned directly at 416 nm. (B) Electrophoresis of 2.9 nmol of hemeprotein in presence of 30 mM cyanide. Gel was scanned at 430 nm, the absorbance maximum of the cyanide complex. (C) The same gel as in B, scanned at 280 nm.

The formylhemin and the protohemin derived from the other hemeproteins tested were shown to be undegraded by spectral and chromatographic techniques as follows. When gels had been treated with thioglycollate and subjected to preelectrophoresis, the pyridine hemochrome of the isolated prosthetic group of the erythrocyte formylhemeprotein was spectrally identical to the pyridine hemochrome of the hemeprotein as applied to the gel. Even without thioglycollate treatment, the hemins isolated from the other hemeproteins were identical to authentic protohemin as assessed by pyridine hemochrome spectra and migrated with protohemin on paper chromatography using 2,6-lutidine/water (10:7) saturated with ammonia vapor (17). This lack of degradation, perhaps a result of low reactivity of protohemin to free radicals, low concentrations of free radicals, or protection by cyanide or urea against chemical modification, allows this technique to be used for the small scale preparation of unaltered

hemins for further analytical work on hemeproteins of unknown prosthetic group.

DISCUSSION

Hemins have traditionally been prepared on a large scale by dissociating the holoprotein into heme and apoprotein by addition of acid, the protonated heme then being removed by extraction into organic solvent. However, the acid and solvent are known to result in modification of some hemes and apoproteins. This is a particularly serious problem for heme *a*, the formyl-containing prosthetic group of an erythrocyte hemeprotein, and other acid-labile prosthetic groups. The formyl group of heme *a* is known to condense with acetone in acid (18). Moreover, there is no analytical procedure in the literature for the isolation of hemins from hemeproteins on a microscale.

The electrophoretic technique presented in this paper avoids the use of extremes of acid or alkali and the use of organic solvent. In this technique the natural ligands of the ferrihemeprotein (at least in the case of hemoglobin, it appears that the heme must be in the ferric form) are replaced by cyanide in slightly alkaline solution. The cyanide plays multiple roles; in addition to replacing the protein's ligands to the hemin it increases the water solubility and electrophoretic mobility of the hemin by adding additional negative charge.

A high concentration of urea must be used in addition to the cyanide in order to release protohemin from methemoglobin, metmyoglobin, horseradish peroxidase, and erythrocyte cytochrome b_5. We assume that urea acts by altering the conformation of the protein thereby disrupting the hemin–protein interactions. By the same token the protein's conformation plays an important role in binding the heme. In contrast, the isolation of the formylhemin is effected by cyanide alone, no urea being necessary. Therefore it is concluded that either protein conformation plays less of a role in binding the formylhemin to its protein or the native forms of the protohemin-containing proteins prevent the cyanide from displacing the hemin.

In the described procedure, released hemin is separated from the protein by discontinuous polyacrylamide gel electrophoresis. The hemin moves as a very sharp band at the chloride–glycinate front in the same fashion that tracking dye or other low molecular weight anions migrate during electrophoresis on disc gels (14, 19). Electrophoretic migration of larger moieties such as the apoprotein is sufficiently retarded by the polyacrylamide matrix so that protein is separated from the hemin by a gentle sieving effect. The sharpness of the resultant hemin band allows clean separation of the hemin from protein and also makes subsequent elution in a small volume feasible with little loss of hemin. The described technique is also useful for the isolation of the unaltered apoprotein form of formylhemeprotein free of hemin and free of hemeprotein. The slightly alkaline conditions used throughout the procedure eliminate the possibility of acid destruction of asparagine, glutamine, and other acid-labile residues and the possibility of cyclization reactions suspected to occur in acid conditions such as those of acid/acetone extraction. These conclusions are supported by our demonstration that the apoprotein of the erythrocyte hemeprotein prepared by this procedure gave a strong, single precipitin band when diffused against antibody prepared against the hemeprotein. One drawback to using this procedure for the preparation of apoprotein from the other hemeproteins is the high probability that the presence of urea would alter the higher order structure of any protein prone to denaturation. However, no caveat appears to be necessary if the apoprotein preparation were for the purpose of performing an amino acid analysis or sequencing, since these procedures have been used for proteins eluted from polyacrylamide gels (20).

It should be stressed that this procedure is not simply a complete dissociation of the hemin from the protein followed by the separation of the two. Rather there is an equilibrium between the hemin–protein complex and the dissociated form. The cya-

nide and urea serve to shift the equilibrium towards dissociation but it is the electrophoretic removal of the hemin that continuously drives this equilibrium towards complete dissociation of hemeprotein. Throughout the electrophoresis, hemin could be seen to leave the protein and collect at the chloride–glycinate front. For these reasons it is probably not feasible to use gel-exclusion chromatography to separate hemin from protein in sharp fractions unless harsher conditions of protein denaturation and ligand replacement are used.

As described, the electrophoretic technique is useful down to about 3 nmol of hemeprotein. When only a few nanomoles of the hemeprotein are obtainable, this electrophoretic procedure would seem to be the method of choice for isolation of the heme for characterization by such procedures as paper chromatography and spectrophotometry. The rapid and gentle nature of the entire procedure makes possible isolation of protoheme and, for the first time, the prosthetic group of the erthrocyte formylheme. Both hemins are obtained in good yield and in apparently undegraded form.

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