Aasa, R., Malmström, B. G., Saltman, P., and Vänngård, T. (1963), *Biochim. Biophys. Acta 75*, 203.
Beinert, H., Griffiths, D. E., Wharton, D. C., and Sands, R. H. (1962), *J. Biol. Chem. 237*, 2337.
Beinert, H., and Palmer, G. (1964), *J. Biol. Chem. 239*, 1221.
Brill, A. S., Martin, R. B., and Williams, R. J. P. (1964), *in* Electronic Aspects of Biochemistry, Pullman, B., Ed., New York, N. Y., Academic Press, p 519.
Bryce, G. F. (1966), *J. Phys. Chem. 70*, 3459.
Carrico, R. J., and Deutsch, H. F. (1969), *J. Biol. Chem. 244*, 6087.
Ehremberg, A., and Yonetani, T. (1961), *Acta Chem. Scand. 15*, 1071.
Falk, K. E., Freeman, H. C., Jansson, T., Malmström, B. G., and Vänngård, T. (1967), *J. Amer. Chem. Soc. 89*, 6071.
Finazzi Agrò, A., Rotilio, G., Avigliano, L., Guerrieri, P., Boffi, V., and Mondovì, B. (1970), *Biochemistry 9*, 2009.
Goa, J. (1953), *Scand. J. Clin. Lab. Invest. 5*, 218.
Gould, D. C., and Mason, H. S. (1967), *Biochemistry 6*, 801.
Gurd, F. R. N., Falk, K. E., Malmström, B. G., Vänngård, T. (1967), *J. Biol. Chem. 242*, 5724.
Kosower, E. M. (1968), Physical Organic Chemistry, New York, N. Y., Wiley, p 179.
Malkin, R., Malmström, B. G., Vänngård, T. (1968), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 1*, 150.
Malmström, B. G., Reinhammar, B., and Vänngård, T. (1968), *Biochim. Biophys. Acta 156*, 67.
Malmström, B. G., Reinhammar, B., and Vänngård, T. (1970), *Biochim. Biophys. Acta 205*, 48.
Malmström, B. G., and Vänngård, T. (1960), *J. Mol. Biol. 2*, 118.
Mason, H. S. (1966), *in* Biochemistry of Copper, Peisach, J., Aisen, P., Blumberg, W. E., Ed., New York, N. Y., Academic Press, p 472.
McCord, J. M., and Fridovich, J. (1969), *J. Biol. Chem. 244*, 6049.
Mondovì, B., Rotilio, G., Costa, M. T., Finazzi Agrò, A., Chiancone, E., Hansen, R. E., and Beinert, H. (1967), *J. Biol. Chem. 242*, 1160.
Peisach, J., Levine, W. G., Blumberg, W. E. (1967), *J. Biol. Chem. 242*, 2847.
Rist, G. H., Hyde, J. S., and Vänngård, T. (1970), *Proc. Nat. Acad. Sci. U. S.* (in press).
Tang, S. P. W., Coleman, J. E., and Myer, Y. P. (1968), *J. Biol. Chem. 243*, 4286.
Warner, R. C., and Weber, I. (1953), *J. Amer. Chem. Soc. 75*, 5094.
Wiersema, A. K., and Windle, J. J. (1964), *J. Phys. Chem. 68*, 2316.
Windle, J. J., Wiersema, A. K., Clark, J. R., Feeney, R. E. (1963), *Biochemistry 2*, 1341.
Wishnia, A., Weber, I., and Warner, R. C. (1961), *J. Amer. Chem. Soc. 83*, 2071.

Chromatographic Isolation and Characterization of Isolated Chains from Hemoglobin after Regeneration of Sulfhydryl Groups*

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ABSTRACT: The chromatographic separation of isolated hemoglobin α^{SH} from α^{PMB} chains and of β^{SH} from β^{PMB} chains is reported.

The purity of the separated compounds was tested by electrophoresis, spectrophotometry, and atomic absorption analysis. Their isoelectric points and the isoelectric points of a number of other hemoglobin derivatives has been determined by ion focusing; the results are consistent with expectations based on the chemical differences between the various hemoproteins.

Isolated chains from hemoglobin in their native state are commonly obtained by reacting hemoglobin at pH 6 with excess PMB[1] (Bucci and Fronticelli, 1965). This method leads to dissociation of hemoglobin into α and β chains which have their sulfhydryl groups reacted with PMB. They can be isolated chromatographically. Regeneration of free SH groups in position 104 on α chains and positions 93 and 112 on β chains has been achieved by several methods (Bucci and Fronticelli, 1965; Tyuma *et al.*, 1966; De Renzo *et al.*, 1967; Geraci *et al.*, 1969); however, none of these methods removes PMB completely without partial denaturation of the protein. Since in many instances it may be crucial to obtain a homogeneous material, chromatographic separation of chains with free SH groups from PMB chains appeared to provide the best solution to the problem.

The procedure is particularly valuable for the detection of small differences in the functional properties of α and β chains, when minor contamination with PMB-containing chains may distort results in view of the rather large effect that the presence of mercurials may have on affinity and reaction rates in the combination with ligands (Antonini

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[1] Abbreviation used is: PMB, *p*-mercuribenzoate.

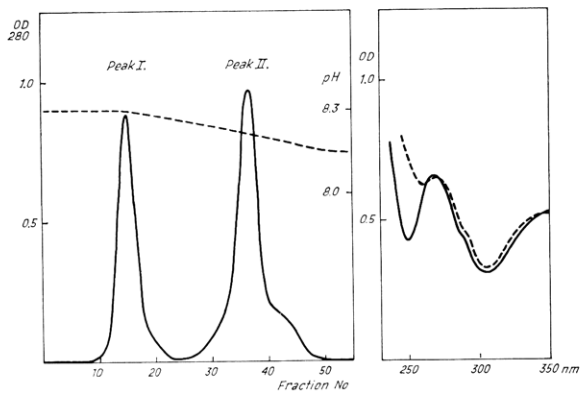

FIGURE 1: Left panel, chromatogram of a mixture of α^{SH} and α^{PMB} chains; for details, see Methods. Right panel, spectra of peak I (————), α^{SH} and peak II; (----), α^{PMB}.

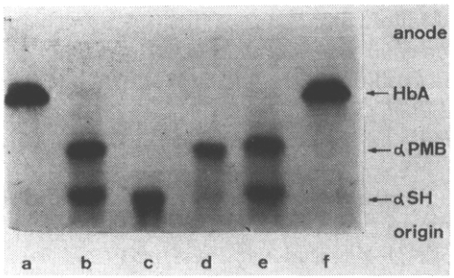

FIGURE 2: Stark gel electrophoresis, pH 8.6, discontinuous buffer system, Amido Black stain. Channels a and f, HbA; channels b and c, mixture of α^{SH} and α^{PMB}; channel c, peak I; channel d, peak II.

et al., 1965; Brunori *et al.*, 1966). This method has also been of great advantage in the preparation of oxidation intermediates as the ones described recently (Brunori *et al.*, 1970).

Materials and Methods

Hemoglobin was purified as previously described (Winterhalter and Huehns, 1964). HbA_2 was isolated on a DEAE-Sephadex column identical with the one used for the separation of α^{SH} and α^{PMB} chains. Globin was prepared as previously described (Winterhalter and Huehns, 1964). IC_{II} of HbA and A_2 were prepared as reported recently (Winterhalter, 1966).

PMB-reacted chains in either the oxy or the carbonmonoxy form were obtained by a method very similar to the one of Bucci and Fronticelli (1965) including minor modifications. Since this procedure gave high yields and is somewhat simpler, the details are given. All operations were carried out at 4°. All buffers used contained 10^{-4} M EDTA. Solutions of hemoglobin with a concentration of 3–8% were dialyzed against 0.1 M phosphate buffer, pH 6.0, containing 0.2 M NaCl. PMB was dissolved in 0.1 M NaOH (about 5 drops/mg) and the solution diluted with 0.01 M phosphate buffer to a final concentration of 1–2 mg of PMB/ml. This mixture was immediately added to the hemoglobin solution to give a final ratio of 7.5 M PMB/mole of Hb (10 mg of PMB/220 mg of Hb). If necessary the pH of the hemoglobin PMB mixture was again adjusted to pH 6.0 with 1 M NaH_2PO_4. After standing for 16–20 hr occasionally forming precipitates were removed by centrifugation for 15 min at 10,000*g*. The resulting clear solution was dialyzed against several changes of 0.01 M phosphate buffer, pH 6.8.

Separation of the PMB-reacted chains was carried out on CM-Sephadex equilibrated with 0.01 M phosphate buffer, pH 6.8. For large batches (4–5 g) columns of 25-mm diameter and 450-mm length were used. For 1 g a column of 20 × 200 mm was sufficient. As little as 15 mg of Hb was successfully separated into chains on a 6 × 35 mm column. Elution was carried out by a gradient obtained by two symmetrical flasks, the first containing the above buffer and the second 0.02 M Na_2HPO_4. The volumes used in each flask were 500 ml for the 20 × 200 mm column and 1000 ml for the large columns. Flow rates were for the small column, 30–40 ml/hr; for the large column, 40–60 ml/hr. The fractions were analyzed for purity by starch gel electrophoresis. Subsequently PMB was removed with dodecanthiol (De Renzo *et al.*, 1967).

Mixtures of α^{SH} and α^{PMB} chains (200–500 mg) were dialyzed against 0.05 M Tris-HCl, pH 8.3, and then loaded onto a DEAE-Sephadex column (25 × 320 mm) equilibrated with the same buffer. The column was developed by a gradient obtained from two symmetrical containers, the first filled with 1000 ml of the equilibrating buffer the second with 1000 ml of 0.05 M Tris-HCl, pH 6.5. Only samples without detectable formation of the ferri derivative were used for further experiments.

Mixtures of β^{SH} chains and β^{PMB} chains were dialyzed against 0.05 M Tris-HCl, pH 8.0, and then placed onto a column (25 × 320 mm) of DEAE-Sephadex equilibrated with the buffer used for dialysis. This column was eluted with a gradient obtained from two symmetrical recipients both filled with 600 ml of 0.05 M Tris-HCl, pH 8.0, but the second additionally containing 0.5 M NaCl.

Purity of the eluted proteins was tested by starch gel electrophoresis at pH 8.6 in a discontinous buffer system (Poulik, 1957). The same method was applied to check recombination with partner chains. The absence of ferri chains was verified spectrophotometrically.

Spectra were recorded on a Cary Model 15 spectrophotometer.

Mercury determinations were carried out by a flameless atomic absorption procedure according to Brandenberger and Bader (1968). The protein solutions containing mercury were diluted 1:20 with distilled water, 5 ml of this dilution was acidified to 1.0 N with HNO_3. The mercury was assayed by electrolytic deposition on a copper wire and evaporated in the atomic state in the light beam of a mercury holocathode lamp. The mercury concentration was determined by the absorption at the 253.7-nm mercury line. The mercury-poor solutions were used in an identical procedure but in a 1:5 dilution.

Determinations of isoelectric points were done on the LKB 8100 electrofocusing equipment using the appropriate ampholyne mixtures to ensure positioning of the protein bands outside the area of high sucrose concentration.

Results

α Chains. The chromatogram resulting from α chain separation is illustrated on the left panel of Figure 1 demonstrating a clear separation of α^{SH} chains (peak I) and α^{PMB} chains (peak II). The right panel of Figure 1 illustrates the spectra obtained from the two separated peaks. Atomic absorption for mercury gave a molar ratio of mercury to heme of 0.91 for the component corresponding to peak II and of about 3 × 10^{-3} for that corresponding to peak I, thus indicating that the latter is virtually free of PMB. Further-

TABLE I: Isoelectric points of Hemoglobin Derivatives.

Sample	No. of Runs	Isoelectric Point	Range
α^{SHO_2}	4	7.82	7.80–7.86
α^{PMBO_2}	3	7.49	7.47–7.50
β^{SHO_2}	3	6.74	6.72–6.74
$\beta^{PMB\text{-}SHO_2}$[a]	1–2	6.38, 6.30	6.36–6.42, 6.30
$\beta^{(PMB)_2O_2}$	6	6.08	6.02–6.12
HbA O_2	5	7.15	7.14–7.15
HbA CO[b]	2	7.22	7.20–7.25
Hb^+ CN	2	7.20	
Globin[c]	3	7.79	7.76–7.82
IC_{II}HbA	3	7.56	7.54–7.58
HbA_2O_2	1	7.62	
$IC_{II}HbA_2$	2	7.88	7.85–7.92

[a] The $\beta^{PMB\text{-}SHO_2}$ peak regularly gave a split band, the major component having an isoelectric point of 6.30 and a minor band with an isoelectric point of 6.38. [b] No resolution was obtained from a 1:1 mixture of HbCO and Hb^+ CN. [c] This value was obtained only when globin focused in an area with low sucrose concentration. Higher values resulted when globin migrated into areas of high sucrose concentration.

more addition of PMB to peak I in a molar ratio of 1 yielded results closely similar to the ones of peak II. Also the electrophoretic mobility of α^{SH} chains after addition of PMB was identical with that of peak II. The relative electrophoretic mobilities of α^{SH} chains and α^{PMB} chains from peaks I and II are illustrated in Figure 2. The isoelectric points of α^{SH} and α^{PMB} chains are summarized in Table I. The actual results reported refer to the oxy derivative, but did not significantly differ from those obtained with the CO derivative.

β Chains. Three different types of chains could be separated and on the basis of mercury analysis they could be identified as $\beta^{(PMB)_2}$, $\beta^{PMB\text{-}SH}$, and $\beta^{(SH)_2}$. The $\beta^{(PMB)_2}$ chains were eluted on the CM-Sephadex column used for chain separation ahead of bulk of β chains. Their spectrum is illustrated in the right panel of Figure 3. The chromatogram obtained from mixtures of $\beta^{PMB\text{-}SH}$ and $\beta^{(SH)_2}$ chains is illustrated in the left panel of Figure 3. The spectra of the two clearly separated proteins are given in the right panel of the same Figure. Atomic absorption determination gave a molar ratio of mercury to heme of 0.97 for peak II and 1.2×10^{-3} for peak I.

Mixtures of $\beta^{(PMB)_2}$, $\beta^{PMB\text{-}SH}$, and $\beta^{(SH)_2}$ were analyzed by electrofocusing and the resulting bands checked by spectrophotometry. The isoelectric points thus obtained are given in Table I. No significant differences between the O_2 and CO derivative could be detected. The same table gives isoelectric points of a number of other hemoglobins derivatives for comparison.

Discussion

As clearly demonstrated in Figures 1 and 2 α^{SH} chains and α^{PMB} chains can be completely separated by the described chromatographic procedure. The α^{SH} chains were shown to be free of PMB by atomic absorption measurement of mercury.

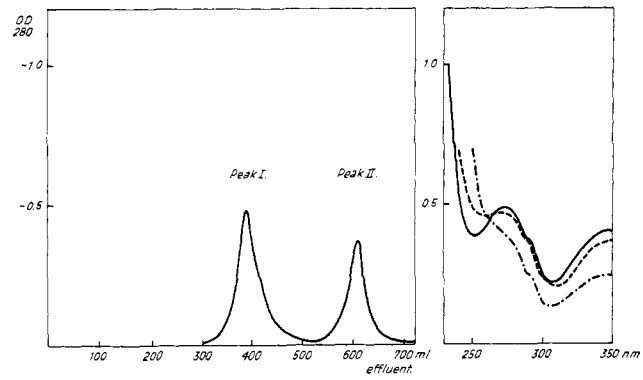

FIGURE 3: Left panel, chromatogram of a mixture of β^{SH} and β^{PMB} chains; for details, see Methods. Right panel, spectra of β^{SH}, peak I (—); β^{PMB}, peak II (----); and $\beta^{(PMB)_2}$ (–·–·–).

The situation with the β chains is somewhat more complicated since they contain cysteine residues at positions 93 and 112 and can therefore have the following compositions: $\beta^{(SH)_2}$, $\beta^{93\text{-}PMB,112\text{-}SH}$, $\beta^{93\text{-}SH,112\text{-}PMB}$, and $\beta^{(PMB)_2}$. Since $\beta^{(PMB)_2}$ chains are mostly monomeric whereas β^{SH} chains tend to form tetramers (Antonini *et al.*, 1965), it is conceivable that tetramers with different kinds of chains, *i.e.*, β^{SH} and β^{PMB} might also exist. However, this seems unlikely in view of the mercury heme ratios for the three separated components; in addition, the β^{PMB} peak was homogeneous even in rechromatography. The peak corresponding to the compound with heme ratio of nearly one probably contains $\beta^{93\text{-}PMB,112\text{-}SH}$ and $\beta^{93\text{-}SH,112\text{-}PMB}$ which are not separated by ion-exchange chromatography. This idea is supported by the observation that $\beta^{PMB,SH}$ chains always gave a split band in the electrofocusing column with two slightly different isoelectric points (Table I) probably corresponding to $\beta^{93\text{-}PMB,112\text{-}SH}$ and $\beta^{93\text{-}SH,112\text{-}PMB}$ chains. In any event purification of PMB-free β chains is readily achieved by column chromatography.

The method presented here permits to obtain hemoglobin chains with fully regenerated SH groups without the need prolonged or drastic removal of PMB which often leads to partial denaturation of the protein. When mixed these chains rapidly and completely combined to form HbA.

The differences in the 274:250 absorption ratios between α^{SH} chains (1.53) and β^{SH} chains (1.3) might be consistent with the higher content of cysteine and phenylalanine of the β chains (α = 7 Phe, 1 Cys; β = 8 Phe, 2 Cys).

The difference in the isoelectric points between PMB and SH chains can be accounted for on the basis of their titration curves by the introduction of extra negative charge (charges), due to the fully ionized carboxyl group of PMB. This suggests that no major conformational change involving ionizing amino acid side chains occurs upon substitution of SH groups by PMB.

The differences in isoelectric points between α^{SH}, β^{SH}, globin, and hemoglobin are at least quantitatively, all consistent with their titration curve and reflect changes associated with removal of heme or partner chains (Bucci *et al.*, 1968).

The isoelectric point of IC_{II} in which only α chains carry heme is intermediate between that of globin and hemoglobin (Winterhalter and Deranleau, 1967).

Finally the difference between HbA and A_2 in line with the different amino acid composition of β and δ chains.

Acknowledgments

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References

Antonini, E., Bucci, E., Fronticelli, C., Wyman, J., and Rossi-Fanelli, A. (1965), *J. Mol. Biol. 12*, 375.

Brandenberger, G., and Bader, H. (1968), *At. Absorption Newslett. 7*, 53.

Brunori, M., Amiconi, G., Antonini, E., Wyman, J., and Winterhalter, K. H. (1970), *J. Mol. Biol. 49*, 461.

Brunori, M., Noble, R. W., Antonini, E., and Wyman, J. (1966), *J. Biol. Chem. 241*, 5238.

Bucci, E., and Fronticelli, C. (1965), *J. Biol. Chem. 240*, 551.

Bucci, E., Fronticelli, C., and Ragatz, B. (1968), *J. Biol. Chem. 243*, 241.

De Renzo, E. C., Ioppolo, C., Amiconi, G., Antonini, E., and Wyman, J. (1967), *J. Biol. Chem. 242*, 4850.

Geraci, G., Parkhurst, L. J., and Gibson, Q. H. (1969), *J. Biol. Chem. 244*, 4664.

Poulik, M. D. (1957), *Nature (London) 180*, 1477.

Tyuma, I., Benesch, R. E., and Benesch, R. (1966), *Biochemistry 5*, 2957.

Winterhalter, K. H. (1966), *Nature (London) 211*, 932.

Winterhalter, K. H., Amiconi, G., and Antonini, E. (1968), *Biochemistry 7*, 2228.

Winterhalter, K. H., and Deranleau, D. A. (1967), *Biochemistry 6*, 3136.

Winterhalter, K. H., and Huehns, E. R. (1964), *J. Biol. Chem. 239*, 3699.

ε-(γ-Glutamyl)lysine Cross-Linkage in Citrulline-Containing Protein Fractions from Hair*

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ABSTRACT: The presence of ε-(γ-glutamyl)lysine as a cross-link in citrulline-containing protein fractions of medulla cells of hair and quill and the inner root sheath cells of hair follicles has been demonstrated by both chemical and enzymic methods. The chemical method determines the number of ε-amino groups of lysines that do not react with acrylonitrile under vigorous conditions. In the enzymic method the cross-link is measured directly by amino acid analysis of complete enzymic digests. Good agreement has been obtained between the two methods. As much as 13 moles of ε-(γ-glutamyl)-lysine/1000 moles of amino acid residues is present in the hair medulla protein. Lesser amounts are detected in the other proteins. The nature of the cross-link has been confirmed by isolation of the new compound appearing in a complete enzyme digest of hair medulla protein and its characterization as ε-(γ-glutamyl)lysine.

The proteins from the cells of inner root sheaths of hair follicles and medullae from hair and related structures such as porcupine quills have been shown to contain citrulline (Rogers, 1962, 1964; Bradbury and O'Shea, 1969). The cells in these tissues (Figure 1) are packed with the respective proteins as are the cortical cells with keratin. Recent work including limited amino acid sequence studies of peptides from porcupine quill medulla has demonstrated that the citrulline is covalently bound in peptide linkage in the proteins (Steinert *et al.*, 1969). In addition to containing citrulline these proteins are characterized by a very high glutamic acid content and a very low cystine–cysteine content and are therefore quite distinct from the surrounding keratin (Rogers, 1962).

The study of these proteins has consistently demonstrated their extreme insolubility in the usual protein solvents, including those used to extract keratin (Matoltsy, 1953; Rogers, 1964). In fact, methods for preparing medullary cells have relied upon this insolubility and have involved drastic conditions to remove the keratin (Matoltsy, 1953; Bradbury and O'Shea, 1969). Further, medullary cells that have been obtained from guinea pig hair using mild methods for the dissolution of the cortical keratin (Gillespie, 1964; Maclaren and Kilpatrick, 1969) have recently been shown to resist solution even under vigorous conditions such as extremes of pH and salt concentration, high concentrations of dissociating agents (urea and guanidine hydrochloride), reducing and oxidizing conditions, organic solvents, and detergents. Indeed, only reagents which cause peptide-bond cleavage, *e.g.*, dilute acid hydrolysis, will dissolve the protein even after the cell membrane has been disrupted (unpublished experiments).

The proteins are, however, readily solubilized by proteolysis (Stoves, 1945; Rogers, 1964), again in contradistinction to keratin. Rogers (1962, 1964) has taken advantage of this phenomenon and developed a method employing digestion with crystalline trypsin to release the proteins. The resultant digest contains a complex mixture of tryptic polypeptides which, although water soluble, have not proved to be very

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