ACTA CHEMICA SCANDINAVICA 7 (1953) 1407—1412

Isolation and Properties of Crystalline Fe-Transferrin from Pig's Plasma

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A method for the isolation of crystalline β_1-metal-combining pseudoglobulin *(siderophilin, transferrin)* from blood plasma has been described by Koechlin [1], who used the plasma fractionation system of Cohn *et al.*[2,3]. The final product is obtained "in a mainly iron-free state" and is crystallisable.

The reactive groups responsible for the affinity of the transferrin for iron are fairly labile. The iron-binding capacity of the transferrin is therefore often reduced during the isolation. When transferrin forms an iron complex (Fe-transferrin), the reactive groups are protected and more stable. It was mainly this stabilisation that suggested the development of the method [4] for isolating iron-saturated transferrin (Fe-transferrin). This isolation procedure differs from that described by Koechlin, this difference being ascribable mainly to the fact that Fe-transferrin is more soluble than transferrin in strong salt solutions and in water-alcohol solutions of low ionic strength. It was later found that Fe-transferrin is fairly resistant to chloroform-alcohol solutions. This resistance has been utilised for eliminating those proteins which even in low concentration inhibit crystallisation of the Fe-transferrin. Satisfactory crystallisation is achievable only if at least 95 per cent of the final product consists of Fe-transferrin.

EXPERIMENTAL

Purification procedure*

I. Fe^{++} (Mohr's salt) is added to pig's serum (6 mg Fe/litre), the amount of iron being slightly in excess of the specific iron-binding capacity of the serum.

II. Fe-transferrin is precipitated with ammonium sulphate (60—72 % saturation).

III. The major part of the albumin is precipitated at pH 5.2 with 20 % by volume alcohol (low ionic strength, + 4°). In order to prevent partial dissociation of the Fe-transferrin, 1 mg of Fe^{++} (Mohr's salt) is added for every litre of protein solution. The Fe-transferrin is now in the supernatant after centrifugation.

IV. The concentration of alcohol in the supernatant layer is increased to 22 % by volume, and the temperature is lowered to −12° C. The precipitate is separated the following day. The precipitate, of which about 10 % consists of transferrin and the remainder of albumin, is dissolved in water and dialysed for 24 hours against running tap water. The dialysed solution is called fraction 1.

* Steps I—VI have been described in detail earlier [4].

V. The concentration of the alcohol in the supernatant is increased to 25 % by volume. The precipitate obtained after the mixture has been allowed to stand for 24 hours at −12° C is separated by centrifugation (−12° C). Some 30−50 % of the precipitate consists of Fe-transferrin. The precipitate is dissolved in water and dialysed for 24 hours against running tap water. The dialysed solution is called fraction 2.

VI. The concentration of the alcohol in the supernatant layer is increased to 28 % by volume. The precipitate consists of Fe-transferrin, a β_1-globulin with haemochromogen-like spectrum and a α_1-globulin. The precipitate is dissolved in water and dialysed for 24 hours against running tap water. The dialysed solution is called fraction 3.

VII. The Fe-transferrin can be isolated from fractions 1, 2 and 3 after precipitation with chloroform-alcohol in accordance with the procedure described below. The quantitatively and qualitatively best yield is obtained from fraction 2.

The pH of the dialysed protein solution is adjusted to 6.0 with 1 *M* acetate buffer (pH 5.5), and 50 μg Fe++ (Mohr's salt) per g protein is added. The solution is diluted with water until the concentration of the protein is about 4 %, and the solution is cooled down to 0° C.

250 ml chloroform-alcohol solution (1 part chloroform and 9 parts 95 % alcohol) is quickly added to each litre of the protein solution into which it is vigourously stirred. The mixture is centrifuged within 30 minutes at 0° C. Most of the transferrin is now in the supernatant layer. It is cooled to −5° C and a further quantity of 175 ml chloroform-alcohol per litre is gradually added, during which the solution is continuously stirred. The temperature of the solution must not rise above 0° C during the addition of the chloroform-alcohol. The mixture is centrifuged in a refrigerated angle centrifuge (−5° C). The major part of the transferrin is now in the precipitate, which is suspended in water and is immediately centrifuged for 10 minutes at about 20° C. The supernatant layer contains Fe-transferrin (fraction A).

After the last precipitation with the chloroform-alcohol the supernatant layer contains some Fe-transferrin and the main part of the abovementioned protein with haemochromogen-like spectrum. Both are precipitated on addition of 10 % solution of calcium chloride (10 ml/litre) to the supernatant. After the solution has been allowed to stand for 24 hours at −12° C the precipitate is collected by centrifugation at −10° C. The precipitate (fraction B) is suspended in distilled water.

Crystallisation of the Fe-transferrin from fraction A.

Crystalline Fe-transferrin can be obtained from fraction A if at least 90 % of the protein consists of Fe-transferrin. If Fe-transferrin is not present in such concentration, repetition of step VII will usually secure such purity. On careful addition of alcohol to a Fe-transferrin solution of sufficient strength (at least 5 %) and with a pH of 7.5 to 4.5 and low ionic strength at 0° C, Fe-transferrin will crystallise. Crystallisation occurs quickest at pH about 6.

Technique. Fraction A is dialysed for 18−24 hours against running tap water. The pH is adjusted to 6 by careful addition of a 2 % solution of acetic acid. The solution is then cooled to 0° C. Afterwards and during continous stirring 90 % alcohol (−10° C) is added dropwise until a slight cloudiness appears. The solution is then warmed to room temperature (20−22° C), at which the precipitate is re-dissolved. The solution is transferred to a closed vessel and allowed to stand at 4° C. Inspection under a loupe (× 10) will often reveal the formation of crystals within an hour, though sometimes not until the following day. Formation of an amorphous precipitate indicates that too much alcohol had been added or that the fraction was not sufficiently pure. The use of too much alcohol can be remedied by the addition of a little water to the solution. The more homogeneous the preparation the quicker do crystals form.

The yield of crystals increases during the first few days. Crystallisation can be accelerated by increasing the concentration of the alcohol by a couple of per cent after 24 hours. On re-crystallisation the crystals assume a macroscopically visible size within an hour.

On re-crystallisation of Fe-transferrin from more concentrated solutions (at least 10 %) the crystals formed measure several millimetres (Fig. 1). The lower the concentration of the alcohol the slower the crystallisation, but the larger the crystals in the end product.

Fig. 1. Fe-transferrin crystallised in a 0.1 cm thick cuvette at pH 6 from an ethanol-water mixture.

Analysis of re-crystallised Fe-transferrin

The determinations given below refer to analyses of preparations re-crystallised four to six times. The degree of purity of each preparation was checked by paper electrophoresis, performed at pH 8.6 at which each of the preparations showed only a spot corresponding to the position of β-globulins. No other proteins were present in measurable concentration.

The dry weight determinations (drying to constant weight at 105° C) were used as a standard for the calculation of the constants given below.

The nitrogen content, as determined by the method of Hiller, Plazin, & van Slyke [5], was 16.3 %.

The carbohydrate content, as determined by Friedmann's modification [6] of the method described by Sörensen *et al.*, was 1.4 %.

The level of the total lipids was so low that it lay below the range of sensitivity of the method [7] used. The preparations were practically lipid-free, at least less than 0.4 %.

The iron content of seven preparations studied ranged between 0.120 and 0.136 % (average 0.126 %).

The extinction coefficients (E) were determined at those wave lengths at which the maximum absorption was obtained in the visible as well as in the ultraviolet part of the spectrum. All values correspond to the extinction of a 1 % protein solution in a 1 cm cuvette at 20° C.

E (470 mμ, pH 7.0) varied between 0.48 and 0.55; E (278 mμ, 0.1 N HCl) was 11.0; E (280 mμ, pH 7.0) was 13.8 and E (290 mμ, 0.1 N NaOH) was 12.7 immediately after addition of sodium hydroxide and reached a relatively stable level at 13.3 60 minutes later.

DISCUSSION

The method described above is useful for the isolation of a product from which Fe-transferrin can readily be crystallised. The crystals (Fig. 1) may grow to 2—4 mm in length.

The iron content of the final product varied between 1.20 and 1.36 μg Fe/mg protein. The iron content, as calculated on theoretical grounds, was 1.27 (1.24) μg/mg protein, if the molecular weight be taken as 88 000 (90 000) and if two atoms of iron are linked to every molecule of protein. The range of variation in the experimental determinations may be explained by the addition of iron during the isolation procedure in order to prevent dissociation of the

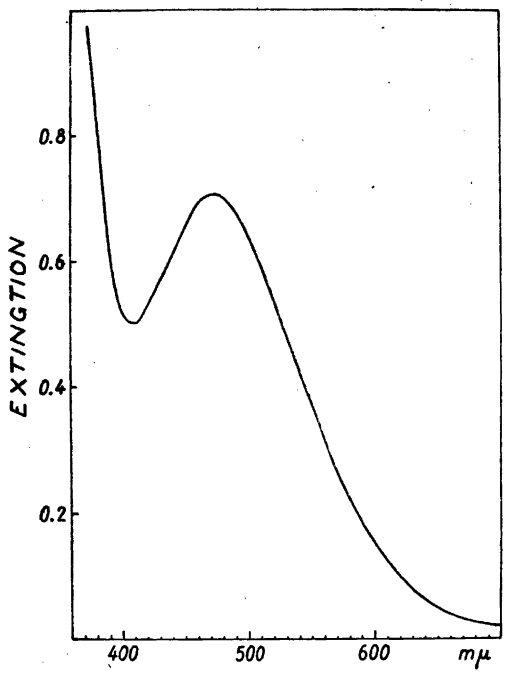

Fig. 2. Light absorption of Fe-transferrin (1.29 %) between 380 and 700 mμ at pH 7.0 (phosphate M/15) (1 cm cuvette).

complex when the pH of the solution fell below 6. Transferrin, just as well as other proteins, is capable of taking up iron in a loose non-specific bond. Unlike the specific Fe-transferrin complex with maximum absorption at 470 mμ, these types of non-specific iron complexes do not absorb any appreciable amount of light in the visible part of the spectrum. The loosely bound iron in one of the preparations was eliminated from the final product by passing the preparation through a column with a cation exchange resin (IRA 120) at pH 7.5. The iron content of the preparation decreased to 1.25 μg per mg protein without any accompanying change in light absorption per mg protein measured at 470 mμ ($E = 0.55$). It may be assumed that the loosely bound non-specific iron was, roughly speaking, quantitatively eliminated by the passage of the solution through the exchange resin. The extinction value at 470 mμ and recalculated per μg iron /E(1μg Fe/ml, pH 7.5) = 0.44/ was slightly higher than that of any of the other preparations. Extinction at 470 mμ per unit of concentration of the iron is the best determination for deciding whether any iron present is specifically bound.

The E-values can be used for checking the purity and integrity of the final product. Values below 0.55 indicate either that the protein is not homogeneous or that the iron-binding groups have been damaged during the isolation procedure, provided the value cannot be increased to 0.55 by the addition of a ferrisalt to the preparation.

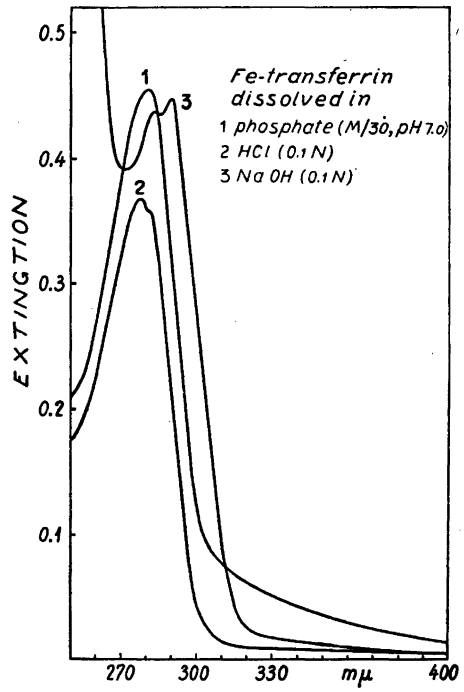

Fig. 3. U.V. light absorption of Fe-transferrin (0.0243 %) in neutral, alcaline and acid medium (1 cm cuvette).

After the Fe-transferrin has been crystallised once it is, as judged by electrophoretic analysis, practically homogeneous, but examination of the light absorption of the preparation in a wave range of 400—500 mμ, will show that such homogeneity is only apparent. Before crystallisation the preparations contain a haemi-globulin presumably formed during the isolation procedure. As far as solubility is concerned, this haemi-globulin resembles Fe-transferrin, but it contains about 10 % carbohydrate. Its electrophoretic mobility lies between that of α_2 and $\beta_1$globulins. It is characterised by very strong absorption of light at 408 mμ and a weaker absorption at 533 mμ. The strongest band falls within a wave range in which the absorption of Fe-transferrin is least (Fig. 2). The purity of the Fe-transferrin, as measured by its absorption spectrum, can thus be checked by the ratio between the absorption at 470 mμ and 408 mμ. Before crystallisation this ratio is usually less than 1, but after the preparation has been crystallized four or five times the ratio is usually 1.40—1.41 and cannot be increased by further crystallisation.

Earlier [8–10] spectra of Fe-transferrin have been illustrated simply as difference spectra between the irony and the iron-free protein, this probably being dictated by the difficulties presented by the quantitative elimination of the haemi-muco-globulin. By using difference spectra the influence of the haemi-muco-globulin is eliminated.

According to Surgenor *et al.* the maximum light absorption by Fe-transferrin is found at 465 mμ and according to Schade [10] at 460 mμ. Both found

E to be 0.57. These values, which were found for transferrin isolated from human serum, agree rather well with those found for pig's serum in the present study (E at 470 mμ = 0.55).

The extinction coefficients for the light absorption of Fe-transferrin with respective maxima in the ultraviolet part of the spectrum are summarised on p. 1409 (Fig. 3).

The absorption at 290 mμ increases during the first hour after the addition of 0.1 N NaOH, after which it reaches a fairly steady level, at which it persists for the few following hours. The light absorption of Fe-transferrin (pH about 13, 280—290 mμ) suggests that the molar ratio between the tyrosine and tryptophane in this protein is 2 : 1.

Comparison of the absorption curves (Fig. 3) distinctly showed that the tyrosine peak was about 25 % higher in neutral environments than in acidic medium. As indicated by the disappearance of the peak at 470 mμ the bond between the iron and the transferrin breaks down in the presence of 0.1 N HCl. The difference between the level of the tyrosine peak in neutral and acid environments may possibly suggest the inclusion of tyrosine in the groups responsible for the specific bond between the iron and the transferrin. In neutral medium the light absorption lies in the region of 300—350 mμ thus likewise strikingly high. This was observed in all of the preparations. Centrifugation for 1 hour at 16 000 r.p.m. produced no change in the shape of the absorption curve. Light absorption at 300—350 mμ is probably related to the specific iron bond of the Fe-transferrin because the absorption values decrease on release of iron from Fe-transferrin both in acid and alkaline medium.

SUMMARY

1. A procedure is described for the isolation of Fe-transferrin from pig's serum in a crystalline state.

2. Data are given about the composition and light absorption of re-crystallised Fe-transferrin.

REFERENCES

1. Koechlin, B. A. *J. Am. Chem. Soc.* **74** (1952) 2649.
2. Cohn, E. J., Strong, L. E., Huges, W. L. Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L. *J. Am. Chem. Soc.* **68** (1946) 459.
3. Surgenor, D. M., Strong, L. E., Taylor, H. L., Gordon, R. S. Jr., and Gibson, D. M. *J. Am. Chem. Soc.* **71** (1949) 1223.
4. Laurell, C.-B., and Ingelman, B. *Acta Chem. Scand.* **1** (1947) 770.
5. Hiller, A., Plazin, J., and van Slyke, D. *J. Biol. Chem.* **176** (1948) 1401.
6. Friedmann, R. *Biochem. J. (London)* **44** (1949) 117.
7. Swahn, B. *Scand. J. Clin. & Lab. Invest. In press.*
8. Holmberg, C. G., and Laurell, C.-B. *Acta Chem. Scand.* **1** (1947) 944.
9. Surgenor, D., Koechlin, A., and Strong, L. *J. Clin. Invest.* **28** (1949) 73.
10. Schade, A., Reinhart, R., and Levy, H. *Arch. Biochem.* **20** (1949) 170.

Received September 19, 1953.