RESEARCH COMMUNICATION

On the preparation of β -haematin

G. BLAUER¹ and M. AKKAWI

Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

Synthetic β -haematin is considered to be identical, or at least very similar to, purified malaria pigment. Methods for its preparation use ferriprotoporphyrin IX at acid pH in the presence of acetic acid at different concentrations and degrees of ionization, elevated temperatures and long reaction times. Here we show that certain widely used reaction conditions, involving very high concentrations of acetic acid/acetate mixtures, do not produce substantial amounts of polymeric β -haematin on im-

mediate isolation of the reaction products, but only during prolonged drying of the products at 37 °C after washing with water. Alternative, more convenient methods of preparation of pure β -haematin are suggested.

Key words: ferriprotoporphyrin IX, Fourier-transform IR spectroscopy, polymerization.

INTRODUCTION

It has been known for many years that haemin precipitated from aqueous alkaline solutions by acetic acid, forms insoluble β -haematin (see [1] for a summary of earlier experiments). A proposed structure of β -haematin involved iron–carboxylate bonds between adjacent ferriprotoporphyrin IX (FP) molecules [1]. Later, it was suggested that β -haematin and the malaria pigment were very similar, supported by observations that both are insoluble in some solvents, in contrast with regular haematin [2]. More recently, a polymeric structure involving a single iron–carboxylate bond between adjacent FP molecules was proposed for both the malaria pigment (haemozoin) from *Plasmodium falciparum*, and for synthetic β -haematin [3]. The Fourier-transform infrared (FTIR) spectrum of haemozoin showed characteristic strong bands at 1664 cm⁻¹ and 1211 cm⁻¹, similar to those of β -haematin [3]. This similarity, or even identity, of the malaria pigment with synthetic β -haematin has stimulated research on the chemical preparation of this polymer in recent years (see, for example [4–11]). However, it was claimed [12] that the substance obtained by one of these methods of preparation (4.5 M acetate buffer, pH 4.5, 30 min heating at 60 °C with FP [9]) is different from polymeric β -haematin. Further investigations by Egan et al. [10], using the above conditions [9], again appeared to yield β -haematin. The present report shows that, contrary to expectation, substantial amounts of β -haematin are formed only upon the prolonged drying of the reaction products at 37 °C after washing with water [see 9,10].

MATERIALS AND METHODS

Type I bovine haemin (chloroferriprotoporphyrin IX) was obtained from Sigma. Glacial acetic acid, AnalaR, was purchased from BDH–Merck Ltd. and DMSO (> 99% purity) was from Merck-Schuchardt (Hohenbrunn, Germany). FTIR spectra were measured on a Perkin–Elmer System 2000 instrument, using KBr discs.

RESULTS AND DISCUSSION

Following the procedure for the production of β -haematin, as described by Egan et al. [10] (4.5 M acetate buffer, pH 4.5, 30 min heating at 60 °C with FP), the products were rapidly

dried *in vacuo* at room temperature, instead of over P₂O₅ for 48 h at 37 °C. We obtained a solid material (yield 95%) which showed characteristic, strong IR peaks near 1625 cm⁻¹ and 1224 cm⁻¹, and also exhibiting a shoulder near 1660 cm⁻¹ (see Figure 1, upper curve). A similar type of spectrum has been

Figure 1 FTIR spectra of the product of reaction prepared according to [10], but dried rapidly *in vacuo*

Upper curve, product washed with distilled water only; middle curve, same product washed with water, then with DMSO [7]; lower curve, for comparison, β -haematin (prepared in 3 M acetic acid, pH 3.1, kept for 6 h at 37 °C; see [7]). Arrows indicate the characteristic absorption peaks at specific wavenumbers.

Abbreviations used: FP, ferriprotoporphyrin IX; FTIR, Fourier-transform infrared.

¹ To whom correspondence should be addressed.

observed by us previously for 'regular' (R-) haematin precipitated at pH 5 in the absence of acetic acid [7]. When this material was further purified by treatment with DMSO [7], it yielded about 27% of a solid (calculated on the basis of the first product) with characteristic FTIR peaks at 1648(s) and 1210(m) cm^{-1} , respectively, corresponding essentially to B-haematin [7] (Figure 1, middle curve). All the above IR-spectra and yields were reproducible. It therefore appears that the product obtained under the conditions given in [9,10], except for the drying process, is substantially different from β -haematin (Figure 1, lower curve), presumably containing only a small fraction of the latter.

We have found, however, that if the material prepared according to Egan et al. [10] was dried over P_2O_5 for 48 h at 37 °C [9], an FTIR spectrum corresponding to β -haematin (characteristic IR peaks at 1663 cm^{-1} and 1210 cm^{-1}) was also obtained in the present study (approx. 80% pure). The reaction of β -haematin formation does not progress in 30 min at 60 °C in the presence of 4.5 M acetate buffer at pH 4.5 but, surprisingly, proceeds during the prolonged drying process at 37 °C, although the acetic acid and salts were apparently removed by thorough washing with deionized water [10]. The presence of B-haematin in the first reaction step (see above) may constitute an intermediate stage in β -haematin formation, as suggested previously for analogous systems [7].

It appears that the initial interaction of FP with acetic acid (e.g. prevention of internal hydrogen bonding in FP aggregates [7]), is sufficient to predetermine the following polymerization reaction, leading eventually to β -haematin. This was also indicated in a preliminary experiment by adding acetic acid (final concentration, 6 M) to a solution of FP in aqueous NaOH at 70 °C, and washing the precipitate at room temperature repeatedly with water to a final pH 5.4. After keeping the wet precipitate for 2 h at 70 °C and subsequent washing with DMSO, β -haematin was obtained, despite the apparent absence of acetic acid during heating the precipitate for 2 h at 70 °C. A control experiment, without heating for 2 h at 70 °C, yielded B-haematin.

It is considered desirable to purify the reaction product of polymerization of FP, not only from water-soluble salts or acids, but also from unreacted FP [3,6–8,11]. This latter purification step was apparently omitted in the method reported in [9,10].

For simplification and time saving preparation of pure β -haematin in high yields, we recommend the following procedure (see also [7]): 5.5 mg haemin are dissolved in 0.35 ml of 0.4 M aqueous NaOH. The solution is diluted by the addition of 0.31 ml water, and then 0.34 ml of glacial acetic acid are added (final concentration, 5.9 M) at a constant temperature (37 °C) and under magnetic stirring. The resulting mixture is kept at 37 °C for 2 h without stirring and is then cooled in water. After centrifugation, the supernatant solution is removed and the precipitate transferred quantitatively to a filter (Swinnex 13,

Millipore, containing Whatman filter paper No. 50). Applying a vacuum during filtration, the precipitate is washed exhaustively with distilled water and then with DMSO, until the filtrate remains faintly coloured (the absorbance per 0.2 cm in the Soret region should not exceed about 1). The amount of product dissolved in the total volume of filtrate is estimated to remain below about 5% of the substance. The material remaining on the filter is washed repeatedly with distilled water, then with a small quantity of ethanol and dried *in vacuo* at room temperature to a constant weight. The yield achieved is over 80% β -haematin as verified by FTIR spectra (see, for example, Figure 1, lower curve). An analogous procedure at 70 °C for 2 h yields over 90% β -haematin.

The formation of β -haematin (haemozoin) is inhibited by a number of quinoline-based antimalarial drugs, such as chloroquine or quinidine [7–11]. Complex formation of FP with these drugs was postulated many years ago [13–16]. However, any test of this inhibition should necessarily be performed under conditions producing authentic β -haematin in substantial yield in the absence of the inhibitor. For this purpose, suitable modifications of the recommended procedures for β -haematin production presented above may be used [7].

Additional experimental work on the systems and reactions described above is in progress and will be published in due course.

We thank Dr O. Navon for his aid in the measurement of the FTIR spectra.

REFERENCES

- 1 Lemberg, R. and Legge, J. W. (1949) Hematin Compounds and Bile Pigments, pp. 170–172, Interscience, New York
- 2 Fitch, C. D. and Kanjanangulpan, P. (1987) J. Biol. Chem. **262**, 15552–15555
- 3 Slater, A. F. G., Swiggard, W. J., Orton, B. R., Flitter, D. W., Goldberg, D. E., Cerami, A. and Henderson, G. B. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 325–329
- 4 Bohle, D. S. and Helms, J. B. (1993) Biochem. Biophys. Res. Commun. **193**, 504–508
- 5 Dorn, A., Stoffel, R., Matile, H., Bubendorf, A. and Ridley, R. G. (1995) Nature (London) **374**, 269–271
- 6 Berger, B. J., Bendrat, K. and Cerami, A. (1995) Anal. Biochem. **231**, 151–156
- 7 Blauer, G. and Akkawi, M. (1997) J. Inorg. Biochem. **66**, 145–152
- 8 Dorn, A., Vippagunta, S. R., Matile, H., Bubendorf, A., Vennerstrom, J. L. and Ridley, R. G. (1998) Biochem. Pharmacol. **55**, 737–747
- 9 Egan, T. J., Ross, D. C. and Adams, P. A. (1994) FEBS Lett. **352**, 54–57
- 10 Egan, T. J., Hempelmann, E. and Mavuso, W. W. (1999) J. Inorg. Biochem. **73**, 101–107
- 11 Basilico, N., Pagani, E., Monti, D., Olliaro, P. and Taramelli, D. (1998) J. Antimicrob. Chemother. **42**, 55–60
- 12 Pandey, A. V. and Tekwani, B. L. (1996) FEBS Lett. **393**, 189–192
- 13 Chou, A. C., Chevli, R. and Fitch, C. D. (1980) Biochemistry **19**, 1543–1549
- 14 Fitch, C. D., Chevli, R., Banyal, H. S., Phillips, G., Pfaller, M. A. and Krogstad, D. J. (1982) Antimicrob. Agents Chemother. **21**, 819–822
- 15 Blauer, G. and Ginsburg, H. (1982) Biochem. Int. **5**, 519–523
- 16 Blauer, G. (1986) Arch. Biochem. Biophys. **251**, 315–322