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Problems of haemoglobin freeze-drying: evidence that water removal is the key to iron oxidation

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Formation of methaemoglobin during freeze-drying of oxyhaemoglobin raises the question of the cause and mechanism of the oxidation. Haemoglobin with or without lyoprotector (250 mM glucose or amino acid salt) has been subjected to freeze drying changes in either or both of two constraints—vacuum and rise in temperature. A rise in temperature from −40 to +10 °C had no substantial denaturing effect on haemoglobin whether protected or not. Maintenance of a vacuum over frozen haemoglobin for 18 h often produced subtotal desiccation. Unprotected haemoglobin was partially oxidized (39% MetHb) whereas protected haemoglobin was not (<4% MetHb). Haemoglobin was also dried by rapid dehydration of thin films in a stream of air at room temperature (20 °C). The methaemoglobin content was then 43% whereas the amino acid salt or glucose limited it at 4 and 7%, respectively. Haemoglobin is oxidized, therefore, only because of the removal of water. Protectors, not specific in structure and action, probably work by holding or reinforcing the critical number of hydration layers around haemoglobin.

Freeze-drying oxyhaemoglobin is known to lead to the formation of methaemoglobin, with oxidation affecting about 50% of the haemoglobin (Cannan & Redish 1942); but Farr et al (1947) showed that adding glucose beforehand effectively protected the haemoglobin from denaturation. Since then, various cryoprotectors or lyoprotectors have been discovered, including carbohydrates, amine buffers, amino acids, macromolecules (Labrude & Vigneron 1984); the best of these totally preserve haemoglobin's structure and functional properties. However, both the action of the protectors and the origin and mechanism of the oxidation remain largely unexplained. To date, as far as we know, little research has been carried out with the sole aim of elucidating these problems. Cannan & Redish (1942) showed that haemoglobin could be oxidized by a drying process other than freeze-drying and Pristoupil et al (1980) referred to the importance of the hydration shell.

In an earlier investigation (Labrude et al 1984) we studied the effects of dialysis, and of the haemoglobin concentration and pH of the drying solution, and the changes in the methaemoglobin level after the start of freeze-drying. Since the process of freeze-drying involves freezing and then, simultaneously, a reduction of pressure and an increase in temperature, in the investigation reported here we have subjected haemoglobin solutions to only one of the elements involved in freeze-drying, with and without a protective agent, in order to measure its oxidizing effect on haemoglobin. In the light of the results obtained, and with the same aim, we then dried a series of identical test samples simply by convection.

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MATERIALS AND METHODS

The solution of human haemoglobin was prepared from citrated blood taken three weeks earlier. The red blood cells, separated and rinsed in 150 mM NaCl, were haemolysed in deionized water. The stromata were isolated by two cycles of centrifugation at 25 000*g* for 30 min, with decantation of the haemoglobin solution both times. The resulting solution was dialysed against deionized water at 4 °C for 15 h and centrifuged once more to eliminate the remaining stromata; the haemoglobin concentration was then adjusted to 100 g L^{-1}.

It was used either just as it was, or with an added protector: D-glucose (Prolabo), or L-arginine-L-glutamate, or L-lysine-L-glutamate (Sigma), of similar effectiveness to a concentration of 250 mM (Labrude & Vigneron 1984). This value was chosen because the protection of haemoglobin during freeze-drying is almost complete and the methaemoglobin percentage always very low.

Effects of an increase in temperature

The 10 mL test samples of solution in 100 mL blood tubes were placed on the shelf of a Usifroid SMH 15

(Maurepas) freeze-dryer and frozen to −40 °C. 'Pseudo-freeze-drying' was then begun, with air being allowed to enter the vessel so that no vacuum was formed. The temperature of the product rose to +10 °C in about 3 h.

Effects of a vacuum

This was studied on samples frozen under the same conditions. The apparatus was set to produce a vacuum as in normal freeze-drying (10^{-2} mbar in the vessel, according to the manufacturer). The haemoglobin samples were kept under vacuum for 18 h. The temperature remained below 0 °C and the materials remained frozen.

Drying in the open air

This was achieved by exposing 10 mL samples of haemoglobin solution, in rectangular polymethylmethacrylate trays 215 × 105 mm (liquid layer 0·44 mm deep), at room temperature (20 °C), to the draught produced by a household fan with blades 50 mm long, set 10 cm away. The forced convection (Lefort des Ylouses 1982) was maintained until the sample was completely dry (3 h).

Control experiments were always done at the same time. Samples were analysed immediately after they had been either thawed or totally dissolved back to the initial volume in deionized water or in a 66 mM Sörensen phosphate buffer of pH such that the final pH of the haemoglobin solution was as close as possible to 7·40 for the purpose of recording the dissociation curve. Any insoluble material was removed by centrifugation. The samples were examined macroscopically (for appearance and colour) and were analysed as follows. Methaemoglobin (MetHb) was estimated by the method of Evelyn & Malloy (1938). Oxyhaemoglobin (HbO_2) was measured with an oximeter (OSM2 Hemoximeter, Radiometer), without adjustment for the methaemoglobin level. The dissociation curve of the haemoglobin was recorded on a Hem.O.Scan (Aminco, USA) at 37 °C, Pco_2 5·32 kPa, with the pH as close as possible to 7·40, and the P50 was determined and the Hill coefficient was calculated for the samples dried by convection.

The averages were analysed statistically using Student's *t*-test and were compared with recently prepared untreated haemoglobin and with freeze-dried haemoglobin both with and without protectors. Where results are not given, this is because the analysis of variance (F test) did not permit comparison of the averages.

Since the fundamental aim of this research was to estimate the oxidation of the haemoglobin (methaemoglobin) and not to obtain dry products for measuring their residual moisture, the water content of the materials submitted to the treatments was not measured; but we have already shown (Chaillot 1984) that this is greater in haemoglobin with a protector (glucose: 11·7%, or arginine aspartate: 2·3%) than in the control samples (0·7%). This is also well known from previous investigations (Pristoupil et al 1985, for example).

RESULTS

The results obtained for untreated haemoglobin and for unprotected freeze-dried haemoglobin are given in Table 1. The Hill coefficient for untreated haemoglobin agrees with published figures (Tyuma et al 1973; Versmold et al 1978; Wajcman 1980). For the record, we also give the results of freeze-drying oxyhaemoglobin with D-glucose and an amino acid salt.

Effects of an increase in temperature (Table 2)

There was no major structural change in the iron of the control haemoglobin or in the protected haemoglobin. The relevant statistical comparisons show that: (a) the oxyhaemoglobin saturation in the presence of a protector was better than in the control ($P < 0{\cdot}001$); (b) the percentage of methaemoglobin increased significantly in the control ($P < 0{\cdot}001$), and the protection afforded by the amino acid salt was significant ($P < 0{\cdot}02$); (c) the difference between the control and the glucose-protected samples after treatment was not significant, but the action of the

Table 1. Structural and functional properties of untreated control haemoglobin and of haemoglobin freeze-dried without or with a lyoprotective molecule.

	Met Hb (%)			HbO_2 (%)			Hill's coefficient		
Haemoglobin	n	mean	s.d.	n	mean	s.d.	n	mean	s.d.
Untreated control	9	0·32	0·91	9	97·5	2·52	14	2·94	0·26
Freeze-dried control	49	49·7	7·84	29	56·3	4·84	10	1·71	0·09
Freeze-dried with D-glucose	10	4·26	1·30	10	91·3	0·98	6	2·59	0·17
Freeze-dried with L-arginine-L-glutamate	10	1·97	0·69	10	95·1	0·69	5	3·00	0·19

Table 2. Influence of freezing and thawing from −40 to +10 °C on the properties of haemoglobin.

	Met Hb (%)			HbO_2 (%)		
Haemoglobin	n	mean	s.d.	n	mean	s.d.
Control	20	1·85	0·95	24	96·5	0·89
With D-glucose	13	1·27	0·72	13	98·5	0·58
With L-arginine-L-glutamate	13	1·00	1·02	14	98·6	1·90

Table 3. Influence of vacuum during 18 h on the properties of haemoglobin in presence or absence of a protective molecule.

	Met Hb (%)			HbO_2 (%)		
Haemoglobin	n	mean	s.d.	n	mean	s.d.
Control						
Not desiccated	4	14·1	1·76	4	87·3	2·86
Desiccated	11	39·2	9·40	11	67·2	6·03
D-Glucose						
Not desiccated	8	1·58	1·73	8	96·1	3·51
Desiccated	6	3·95	3·58	6	96·4	3·69
L-Lysine-L-glutamate						
Not desiccated	6	0·73	0·73	6	98·5	0·81
Desiccated	4	1·87	0·78	4	97·4	0·65

glucose was not the same as that of the amino acid ($P < 0·05$).

Effects of a vacuum (Table 3)

Although the samples tested were all the same at the outset, apart from the presence or absence of a protector, some were completely or partially desiccated, while others remained frozen. This was true in all three series of solutions used. Of 15 control samples, about 3/4 were desiccated, whereas the presence of glucose or amino acid salt resulted in most of the materials remaining hydrated (57 and 60%, respectively). All the samples were analysed.

Of the vacuum-treated controls, the samples that remained liquid were significantly altered in comparison with untreated haemoglobin ($P < 0·001$ for the chosen parameters). The desiccated control samples had undergone a greater degree of oxidation into methaemoglobin than those that remained hydrated, and consequently their oxyhaemoglobin level was lower ($P < 0·001$).

Haemoglobin dried solely by vacuum appeared less denatured than the freeze-dried haemoglobin ($P < 0·01$).

When glucose was present the haemoglobin retained its properties, and no significant differences were found between dried and undried samples. They were no different from untreated control haemoglobin. On the other hand, they were less denatured than was the unprotected vacuum-treated haemoglobin (HbO_2, $P < 0·01$; MetHb, $P < 0·001$), especially if this was desiccated.

The raw results were similar when L-lysine-L-glutamate was used as protector. However, although the results for desiccated and non-desiccated products were very similar, there was a significant difference as regards methaemoglobin ($P < 0·02$). Moreover, variations rendered the comparison with similarly treated or untreated control haemoglobin impracticable. This was also true of comparisons between glucose and amino acid salt, whether hydrated or not.

Effect of drying by convection (Table 4)

In conditions of gentle drying, haemoglobin was oxidized into methaemoglobin, and both the protectors used were effective, though less so than in freeze-drying ($P < 0·01$ for HbO_2). The haemoglobin was better protected from change by the amino acid salt than by glucose (HbO_2, $P < 0·01$; MetHb, $P < 0·05$). However, samples containing glucose differed significantly from non-treated controls (HbO_2, $P < 0·01$). This was also true for the amino acid salt, despite the positive results obtained (HbO_2 and MetHb: $P < 0·001$). Although the dried control samples were heavily oxidized with methaemoglobin and contained insoluble substances, the results were significantly different from those for unprotected freeze-dried haemoglobin ($P < 0·01$).

Finally, when open-air drying was compared with the dehydrating effect in a vacuum for each type of sample, further significant differences were found between the control samples (HbO_2, $P < 0·05$), between the samples with glucose (HbO_2, $P < 0·01$), and between the samples containing the amino acid salt (HbO_2, $P < 0·001$). This means that the causes of the oxidation of haemoglobin are not all the same and do not have the same consequences.

Table 4. Influence of ambient air drying on structural and functional properties of haemoglobin.

	Met Hb (%)			HbO_2 (%)			Dissociation curve ($\bar{m}$)			
Haemoglobin	n	mean	s.d.	n	mean	s.d.	n	pH	P50 (kPa)	Hill's coefficient
Control	11	42·6	9.65	11	61·5	5·56	2	7·47	2·62	2·22
D-Glucose	5	7·14	3·03	5	88·8	1·60	2	7·38	3·21	2·77
L-Arginine-L-glutamate	5	3·48	1·83	5	92·3	1·13	2	7·48	3·25	3·02

DISCUSSION

The purpose of this research was to find out what factor or factors cause haemoglobin to oxidize as it dries, and how the protective agents work.

Freeze-thawing, i.e. rewarming the haemoglobin solution, has no noteworthy effect on the oxyhaemoglobin, although there are certain statistically significant changes. The two protectors played only a minor part in this experiment.

The effect of a vacuum was more complicated. Although all the samples were identical and prepared in a similar manner, they did not all react in the same way, and the state of the haemoglobin at the end of the experiment was dependent on those reactions and on the presence of the protective molecule. The difference in number of desiccated samples could be due to a different rate of ice sublimation as a result of the formation of freeze-dried plugs of different composition. In all the controls the oxidation was related to the degree of dehydration. This shows that the oxidation of haemoglobin is caused either by the vacuum or by the loss of water. In the same conditions, the protected samples underwent less dehydration and less oxidation. It can therefore be concluded that the presence of the protector is an obstacle to the loss of water and that this withdrawal results in increased oxidation. This shows that it is the dehydration which denatures the haemoglobin and not the vacuum itself.

To corroborate these results, we dried the same samples by simple convection. The haemoglobin behaved in a similar manner; there was comparable oxidation into methaemoglobin, and protection by the two added protectors. These results agree with the earlier hypothesis, since the same result was achieved by different methods of dehydration. The fact that the protectors were slightly less effective during this kind of drying than during freeze-drying would also seem to confirm that the vacuum does not play a part in the denaturation.

Many water molecules are distributed around the periphery of proteins and are probably held by strong interactions and are therefore less mobile in the vicinity of ionized or polar groups (Simatos 1982). Grösch & Noach (1976) estimated that the maximum number of monolayers within which water molecules have some structure imposed on them by surface effects is 8; this defines the thickness of the hydration shell that directly interacts with the macromolecule. A recent study from our laboratory (Chaillot 1984) showed that oxyhaemoglobin begins to be oxidized when the water content is still high: between 30 and 40 g per g of protein, i.e. an amount 600 times higher than is needed to provide the first monolayer. So oxidation of the haemoglobin occurs even though the protein still has many monolayers of water. This suggests that the water is essential for the stability of both the globin and the haem, since the three very different techniques we have used lead to similar results.

Ondrias et al (1981) reported substantial structural changes in the haem and in the iron/histidine linkage following physical treatment of haemoglobin and suggested that they depend on the interaction with water; water molecules located in the haem pocket interact directly with the haem or the proximal histidine. They also suggest the plausibility of long-range effects, in which the tertiary structure of the haem is affected by hydrogen bond interactions with water at the surface of the protein. It is clear that our dehydration treatment disturbs the hydration layer and probably also the globin (Thirion et al 1983), and so the effects referred to by Ondrias et al could have repercussions in the haem and the proximal histidine, whose essential role is well documented (Perutz 1982).

The effect of the protectors is increasingly considered to be the result of interactions with water. Arakawa & Timasheff (1982) showed that carbohydrates, whose lyoprotective effect is well known (Labrude et al 1980), have both a hydrating and stabilizing effect on proteins. More recently, Hanafusa (1983) put forward the same hypothesis with regard to a number of compounds that we find effective in the freeze-drying of haemoglobin (sucrose, glucose, potassium glutamate). The compounds that protect oxyhaemoglobin from oxidation, such as glucose and amino acid salts, seem to act mechanically, strengthening the hydration layer and the native conformation of the haemoglobin; this reinforcement seems to counteract long-range disturbances of the haem pocket and perhaps also the diffusion of oxygen molecules.

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