Iron Oxidation and Transferrin Formation by Phosvitin

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The catalytic activity of phosvitin in Fe(II) oxidation and the addition of iron to transferrin were studied under various conditions. It was concluded that the Fe(II) oxidized by phosvitin would bind to apotransferrin, although an appreciable fraction of Fe(III) remained bound to phosvitin. Fe(III) also migrated from phosvitin to apotransferrin. This reaction was first-order with respect to Fe(III)–phosvitin concentration with a half-time ($t_{1/2}$) of 10 min, and a first-order rate constant, $k = 0.069 \text{ min}^{-1}$, in $700 \mu\text{M}$ -phosphate buffer, pH 7.2, at 30°C . The catalysis of the oxidation of Fe(II) by phosvitin was proportional to O_2 concentration, and is quite different from the relative O_2 independence of Fe(II) oxidation as catalysed by ferroxidase. A scheme for the mobilization and transfer of iron in the chicken, including the role of ferroxidase, phosvitin and transferrin, is presented.

During a search for iron-oxidizing enzymes other than ferroxidase (EC 1.16.3.1, Fe(II)– O_2 oxidoreductase) in the sera of various animals, we noted a significant transferrin-forming activity in the sera of egg-laying hens. This substance was purified and identified as phosvitin (Sexton, 1974).

The properties of phosvitin have been reviewed extensively by Taborsky (1974). It has a molecular weight of approx. 40000, 12.3% nitrogen and 10% phosphorus (Taborsky & Mok, 1967). Its amino acid composition is unusual in that approx. 60% of the amino acid residues are serine which are almost fully phosphorylated. The presence of appreciable amounts of phosphate results in phosvitin having a strong affinity for bivalent metals such as calcium, magnesium and iron. At low pH iron binds more tightly to phosvitin than to transferrin (Panic, 1970). However, at pH 7.4, iron was reported not to be transferred from transferrin to phosvitin (Ali & Ramsay, 1974).

The interaction of iron with phosvitin was studied by Taborsky (1963). In the presence of O_2 and phosvitin, Fe(II) was rapidly oxidized to Fe(III), which was accompanied by a rapid O_2 uptake. The significance of ferroxidase and ferri-reductase activity with respect to Fe(II)-to-Fe(III) cycles has been reviewed by Frieden (1973) and Frieden & Osaki (1974). Since iron for haemoglobin

biosynthesis is believed to come directly from transferrin, we studied the migration of iron from phosvitin to transferrin and its possible significance in iron metabolism in egg-laying hens.

Experimental

Materials

Phosvitin. Phosvitin from egg yolk was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Ferroxidase. Twice-recrystallized human ferroxidase (EC 1.16.3.1) was obtained from Cohn IV-1 fraction of human serum as described by Osaki *et al.* (1964).

Apotransferrin. Iron-free transferrin (Behringwerk A.G., Marburg/Lahn, Germany) was dissolved in deionized water and dialysed extensively against deionized water (Johnson *et al.*, 1970). An apotransferrin stock solution was prepared at $200 \mu\text{M}$ and stored at 4°C .

Fe(II). A stock solution of Fe(II) ($200 \mu\text{M}$) was prepared by dissolving crystalline $\text{Fe}[(\text{NH}_4)(\text{SO}_4)]_2 \cdot 6\text{H}_2\text{O}$ in distilled deionized water. This solution was stable for more than 3–5 h. More than 97% Fe(II) was detected in the stock solution at the end of each experiment.

Miscellaneous reagents. Where possible all chemicals used were reagent-grade except ascorbic acid which was U.S.P. (Merck, Sharp and Dohme, Rahway, N.J., U.S.A.), and DEAE-Sephadex A-50 from Pharmacia Fine Chemicals, Piscataway, N.J., U.S.A. These included sodium acetate, $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, NaCl , $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, EDTA (disodium salt), $\text{Mg}(\text{NO}_3)_2$ and KCl from J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A., KH_2PO_4 , NaHCO_3 ,

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Fig. 1. Fraction of Fe(III)-transferrin formed or Fe(II) remaining in the reaction mixture observed at four different pH values in 700 μ M-phosphate buffer at 30°C plotted against time

The reaction mixture contained at zero time 700 μ M-phosphate, 44 μ M-apotransferrin, 40 μ M-Fe(II), 1.2 mM-NaHCO₃ and 170 mM-NaCl with (●) or without (○) 80 μ M-phosvitin (in terms of phosvitin phosphate). The solid and broken lines indicate percentage Fe(III)-transferrin formed and percentage Fe(II) remaining respectively. (a) pH 6.3; (b) pH 6.6; (c) pH 6.9; (d) pH 7.2.

Na₂SO₃, $\alpha\alpha'$ -bipyridyl and glucose from Fisher Scientific Co., Fairlawn, N.J., U.S.A., and Trizma hydrochloride and bovine serum albumin (fraction V) from Sigma Chemical Co.

Methods

Phosvitin purification. Commercial phosvitin (Sigma) was chromatographed on DEAE-Sephadex A-50 by the method of Heald & McLachlan (1964);

Table 1. *Percentage oxidation of Fe(II) after 300 s of reaction*

The reaction was carried out in 700 μ M-phosphate buffer at 30°C. When phosvitin was present the phosvitin phosphate concentration was 80 μ M. Percent additional Fe(III) is the remaining Fe(III) not detectable as Fe(III)-transferrin or Fe(II) (estimated as 100.0% less the sum of the first two columns).

Concentration of ferroxidase (nM)	No phosvitin			With phosvitin		
	% as Fe(III)- transferrin	% as Fe(II)	% additional Fe(III)	% as Fe(III)- transferrin	% as Fe(II)	% additional Fe(III)
pH 6.0						
0	20.6	75.2	4.2	28.4	6.8	64.8
20	61.4	30.5	8.1	39.1	1.8	59.1
50	96.8	4.6	-1.4	43.8	1.2	55.0
100	95.8	3.3	0.9	42.2	2.7	54.1
pH 7.2						
0	53.1	16.4	30.5	44.3	1.9	53.8
20	85.9	14.4	-0.3	43.2	2.7	54.1
50	97.9	8.3	-6.2	44.8	1.0	54.2
100	96.8	4.8	-1.6	47.1	1.3	51.6

it was eluted at 0.40M-NaCl (Sexton, 1974; Heald & McLachlan, 1964). Iron-free phosvitin was prepared by dialysis against 10mM-EDTA (Taborsky, 1963).

Fe(III)-transferrin formation. The rate of iron addition to transferrin in 700 μ M-phosphate* buffer was followed spectrophotometrically at 460 nm in a Cary 15 spectrophotometer at 30°C (Osaki *et al.*, 1966). The reaction mixture contained 700 μ M-phosphate, pH 6.0, 6.3, 6.6, 6.9 or 7.2, 44 μ M-apotransferrin, 1% NaCl, 1.2mM-NaHCO₃ and the indicated concentrations of Fe(II), phosvitin or ferroxidase.

Fe(II) determination. Fe(II) remaining in the reaction mixture was detected by adding a sample (200 μ l) of the reaction mixture to 800 μ l of 12mM- $\alpha\alpha'$ -bipyridyl solution. The pink-coloured solutions were measured at 530 nm in a Cary 15 spectrophotometer. The small amounts of absorption at 530 nm contributed by transferrin present in this solution were corrected for in the final value.

Other methods. O₂ uptake was monitored by an O₂ electrode as described by Osaki *et al.* (1966). Phosphorus and protein concentrations were determined by the methods reported by Ames (1966) and Lowry *et al.* (1951) respectively. Phosvitin phosphate was isolated by the method of Greengard *et al.* (1964a). Serum ultrafiltrate was obtained by using a PM-10 membrane (Amicon Corp., Lexington, Mass., U.S.A.).

Results and Discussion

Transferrin formation in the presence of phosvitin

In the present work, we show that the rate of addition of iron to transferrin from Fe(II) and

apotransferrin was stimulated by the presence of phosvitin. However, as discussed below, phosvitin not only oxidizes Fe(II) to Fe(III), but also binds considerable amounts of Fe(III). Therefore it should be emphasized that transferrin formed did not represent the total Fe(II) oxidized.

pH-dependence. Transferrin formation stimulated by phosvitin increased over the pH range 6.0–7.2; however, the relationship is not linear, but slightly concave. At pH 7.2 the activity is approximately three times that at pH 6.0.

Apotransferrin concentration. The initial rate of Fe(III)-transferrin formation at pH 6.0 depended linearly on apotransferrin concentration within the range 11–44 μ M.

Disparity between transferrin formed and Fe(II) oxidized. Fig. 1 shows the time-course of transferrin formation and Fe(II) remaining in the reaction mixture at four different pH values. The amount of transferrin formed and Fe(II) disappearance are in close parity in the absence of phosvitin at pH 6.3–6.6. However, they are not in agreement if phosvitin is included in the reaction mixture. Table 1 records the percentage loss of Fe(II) 5 min after the initiation of Fe(II) oxidation. Thus under these reaction conditions more than 50% of the oxidized Fe(II) did not bind to apotransferrin in the presence of phosvitin, whereas an almost stoichiometric amount of transferrin was formed in the absence of phosvitin. The decreased formation of transferrin in the presence of phosvitin is probably due to the binding of Fe(III) by the phosphate groups of phosvitin. It is also possible, but unlikely, that a part of the oxidized iron did not bind to either apotransferrin or phosvitin. The likelihood that phosvitin is competing for Fe(III) is also supported by the data presented in Fig. 2. A plot of the substrate concentration against

* Average free phosphate concentration of hen's serum is 700 μ M (Biological Handbook, 1961).

Fig. 2. Rates of transferrin formation plotted against initial substrate concentrations

The experiment was carried out in 700 μM -phosphate buffer, pH 6.0, with (●) or without (○) 80 μM -phosvitin. The reaction conditions were the same as in Fig. 1.

Fig. 3. Rate of O_2 uptake at various substrate concentrations and at an O_2 concentration of 236 μM plotted against the initial Fe(II) concentration

The O_2 uptake was measured by an O_2 electrode in a reaction mixture identical with that of Fig. 2. ●, +80 μM -phosvitin; ○, -phosvitin.

the initial rate of transferrin formation gave a sigmoidal curve. When the data were examined in a plot of v versus v/s (see inset of Fig. 2), a marked deviation was observed at lower substrate concentrations. The simplest explanation for these data is that an inhibitor was bound to the substrate (Webb, 1963). Considering the fact that phosvitin not only oxidizes Fe(II) to Fe(III) , but also binds Fe(III) , it is conceivable that, at lower substrate concentrations, less Fe(III) is available to apotransferrin (because of binding to phosvitin), thus inhibiting Fe(III) -

transferrin formation. If this is true, the rate of O_2 uptake due to Fe(II) oxidation by phosvitin would not show the peculiarity which occurred in the direct transferrin measurements.

Fe(II) oxidation monitored by O_2 uptake. Fig. 3 shows that simple Michaelis-Menten-type kinetics are applicable if Fe(II) oxidation is measured by O_2 uptake. A linear plot of v versus v/s is noted in the inset of Fig. 3. The K_m and $V_{\max}$ values estimated for the reaction are $K_m = 144 \mu\text{M}$ [Fe(II)] and $V_{\max} = 49 \mu\text{M/min}$ (O_2) in the presence of 80 μM -phosvitin phosphate at 30°C in 700 μM -phosphate buffer, pH 6.0.

The rate of O_2 uptake is proportional to O_2 concentration in the reaction mixture (Fig. 4). This effect is quite different from the Fe(II) oxidation catalysed by ferroxidase, where the rate of O_2 uptake is constant above 10 μM - O_2 (Osaki *et al.*, 1966). This feature of O_2 -dependence in the phosvitin-catalysed oxidation of Fe(II) resembled the characteristics of Fe(II) autoxidation previously reported. This suggests that phosvitin has no catalytic effect towards Fe(II) oxidation at low O_2 concentrations and therefore is unlikely to be a factor in affecting the Fe(II) -oxidation rate at the micro-capillary level *in vivo*. Thus the probability of iron being mobilized from iron-storage cells by the iron-oxidizing activity of phosvitin is low, because of its high K_m value for Fe(II) and its low sensitivity to O_2 (Osaki *et al.*, 1971).

Stoichiometry between initial Fe(II) concentration and total O_2 uptake. Fig. 5 shows the total O_2 uptake measured 5 min after the initiation of the Fe(II) oxidation at various phosvitin concentrations. The two non-stoichiometric points at the lower

Fig. 4. Rates of O_2 uptake in the presence of 80 μM -phosvitin phosphate and 40 μM - Fe(II) in 700 μM -phosphate buffer, pH 6.0, plotted at various O_2 concentrations

The rates of autoxidation of Fe(II) were subtracted.

phosvitin concentrations were possibly due to the incomplete oxidation of Fe(II). It is noteworthy that in the presence of apotransferrin, the stoichiometry of 1:4 between molecular O_2 and Fe(II) was maintained at the various phosvitin concentrations, whereas without apotransferrin, extra O_2 was taken up. Grant & Taborsky (1966) reported that excessive O_2 uptake occurred during Fe(II) oxidation by phosvitin. They noted that the oxidation generated labile protein-bound phosphate and nitrogen which led to the formation of new carbonyl groups. In the present

Fig. 5. Total O_2 uptake plotted against various phosvitin phosphate concentrations

The reaction mixture was equilibrated with atmospheric O_2 . $\circ$, No apotransferrin added to the reaction mixture. The excessive O_2 uptake is probably due to oxidation of phosvitin itself. Experimental conditions ($30^\circ C$ etc.) are the same as described in Figs. 1–3.

experiment, excessive O_2 uptake was prevented by the addition of apotransferrin. This observation suggests that apotransferrin *in vivo* may protect phosvitin from being oxidized.

Fe(III) transfer reaction between phosvitin and apotransferrin. When transferrin formation from Fe(II) and apotransferrin in the presence of hen serum was tested, the reaction was biphasic. An initial rapid change was followed by a slow but almost linear increase in E_{460} . The test for the presence of residual Fe(II) in the reaction mixture was negative. This slow increase in absorbance was enhanced by the presence of 0–5 mM-phosvitin phosphate or hen serum ultrafiltrate from a PM-10 membrane (mol.wt. cut-off, 10000). It was suspected that Fe(III) was being transferred from Fe(III)–phosvitin to apotransferrin.

A purified phosvitin isolated from chicken egg yolk but without removal of iron by EDTA dialysis was used with apotransferrin in 700 μM -phosphate at 460 nm. Fig. 6(a) shows that transfer of Fe(III) from phosvitin to apotransferrin proceeded in a first-order mode with a half-time, $t_{1/2}$, of 10 min, and a first-order rate constant, $k = 0.069 \text{ min}^{-1}$, suggesting that more than 90% of phosvitin-bound Fe(III) would be transferred to apotransferrin within 40 min. Fig. 6(b) shows a linear pH-dependence for this transfer reaction at pH 6.0–7.2.

Role of phosvitin in iron transport

A possible role of phosvitin in at least the overflow of plasma iron has been suggested by Planas & Frieden (1973). They reported a large increase in serum iron together with an increase in serum ferroxidase activity after injection of diethylstilboestrol into roosters. This synthetic oestrogen is

Fig. 6. First-order plot of iron transfer from phosvitin to apotransferrin (a) and rate of iron transfer from phosvitin to apotransferrin plotted against pH (b)

(a) The percentage remaining phosvitin iron (calculated value from total iron content) is plotted on a log scale against time. The reaction mixture did not contain either Fe(II) or iron-free phosvitin, but contained an iron-loaded phosvitin. The initial phosvitin-bound iron was 58 μM . The reaction was carried out at pH 7.2 in 700 μM -phosphate buffer. (b) The conditions of the experiments were the same as for (a) except for the difference in pH.

Scheme 1. Diagram of possible iron mobilization and transfer in hen plasma

Abbreviations are as follows: Pv, phosvitin; Fox, ferroxidase; aTf, apotransferrin; Fred, ferritin-reducing system; IEC, immature erythroid cells; $t_{1/2}$, half-time for Fe(III)-transfer reaction. The asterisk indicates that the reaction is only feasible at relatively high O_2 concentrations.

known to induce phosvitin when injected into the rooster (Greengard *et al.*, 1964a). The amount of diethylstilboestrol used by Planas & Frieden (1973) was low, approx. 10% of that which induces a massive amount of phosvitin in the cockerel and which can be detected chemically. However, $1\mu M$ -phosvitin which appeared in the serum was easily detected as a ferroxidase activity. Considering the fact that total iron in serum exceeded by more than 50% the total transferrin after injection of diethylstilboestrol, it is possible that the increased ferroxidase activity reported by Planas & Frieden (1973) could be due to newly synthesized phosvitin. Despite the large increase in serum iron (up to $500\mu g/100ml$), Planas & Frieden (1973) found no increase in transferrin. It may also be assumed that part of the phosvitin transported the additional iron.

Scheme 1 shows the mobilization and transfer of iron in egg-laying hens on the basis of data from both present and previously cited experiments. The iron-storage cells include not only those from liver, but also the duodenal cells, where absorbed iron is stored temporarily as ferritin. Fe(II) produced by a ferritin-reducing system (Fred) (Sirivech *et al.*, 1974) may be directly mobilized by the activity of ferroxidase, or the Fe(II) may bind initially to intracellular phosvitin. The Fe(II)-phosvitin complex then migrates to the blood plasma where it can be oxidized in the higher- pO_2 environment of the blood. It is unlikely

that a rapid oxidation of Fe(II)-phosvitin can occur at the low O_2 concentration inside the cell. Fe(III)-phosvitin also may form from the binding of Fe(III) produced by ferroxidase.

These data suggest a role in this scheme for the transfer reaction of Fe(III) from phosvitin to apotransferrin. J. Planas (personal communication) has found that no phosvitin-bound iron can be detected before the saturation of transferrin with iron. As shown in the present experiments a rapid transfer of iron can occur from phosvitin to apotransferrin. In a dynamic equilibrium under conditions *in vivo*, if Fe(III) comes out from a storage cell bound to phosvitin, iron is rapidly transferred from phosvitin to apotransferrin. In an egg-laying hen approx. 1 mg of iron per day is absorbed and transferred to the maturing egg yolk, mostly as phosvitin-bound iron (Greengard *et al.*, 1964b). Considering the body weight of the average hen (1–1.5 kg), this 1 mg of iron outflow to the yolk represents a sizable iron mobilization and transfer. Thus the transfer reaction of Fe(III) from phosvitin to apotransferrin helps to maintain an adequate iron supply for haemoglobin biosynthesis in the hen and egg.

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