The Transfer of Hematin from Horse Myoglobin to Horse-radish Apoperoxidase

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Hematin transfer between hemoproteins in living cells has not yet been demonstrated, but *in vitro* a transfer from *Aplysia* metmyoglobin to horse apomyoglobin occurred within a few hours.[1] Transfers also took place from *Aplysia* metmyoglobin to horse apohemoglobin and from horse methemoglobin to horse apomyoglobin. The reverse reactions could not be detected. The hematin-protein dissociation constants for horse metmyoglobin and human methemoglobin have been calculated from equilibria between hemoprotein and histidylhistidine and between this peptide and hematin.[2] In the present investigation the transfer of hematin from horse metmyoglobin to the apoprotein of horse-radish peroxidase has been studied by spectrophotometric and chromatographic methods and by peroxidase activity measurements. The appearance of peroxidase activity is taken as conclusive evidence for the correct binding of hematin to apoHRP. The ratio between the apparent affinities of hematin for the two protein moieties at pH 10 have been determined.

HRP_C, Mb_1, and their apoproteins were prepared as published.[3-6] The heme-binding capacities of the apoproteins were determined by titration with hematin.[7] Peroxidase activities were assayed by the guaiacol method [8] with a sample of the unsplit HRP as reference. HRP and Mb in the reaction mixture were separated by chromatography on CMC. Aliquots were brought to pH 5.9 and applied onto columns previously equilibrated with 50 mM sodium phosphate of pH 5.9. HRP passed through while Mb was retained and could be eluted with a small volume of 0.2 M K_2HPO_4. The eluted hemoproteins were identified and assayed spectrophotometrically.

* Abbreviations: Mb, Mb^+, Mb·OH and HRP, HRP^+, HRP·OH denote horse myoglobin and horse-radish peroxidase and their acid and alkaline forms.

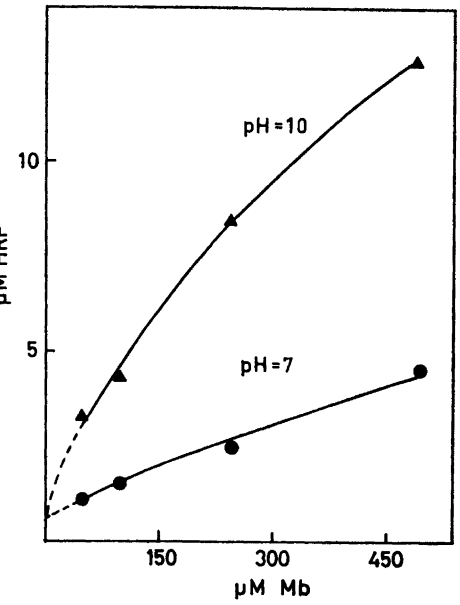

Fig. 1. The formation of HRP from apoHRP (13.4 μM) and Mb. Ordinate: HRP formed in 17 h as found by activity measurements. Buffers: H_3BO_3—NaOH—KCl, pH 10.0, $\mu = 0.1$; sodium phosphate pH 7.0, 20 mM.

The sum of the concentrations of binding sites (free or occupied; one site per molecule) always exceeded the total concentration of hematin, and the amount of free hematin dimer [9] was hence negligible. The reaction times were probably long enough to permit transitory complexes initially formed [10] to disappear, and for the spectrophotometric analyses it was assumed that only two species were present in the solution, Mb and HRP. Storage of Mb for 2 h at pH 6—10 does not change the Soret band,[11] and HRP is probably stable within this range.[12] So far nothing seems to be known about the stabilities of apoMb and apoHRP at pH 10, and our calculations have been made on the assumption that no denaturation took place. All experiments were performed at 25° with the solutions in contact with air.

Fig. 1 compares the migrations of hematin from Mb to apoHRP at pH 7 and 10. A sample of Mb gave only a negligible peroxidase activity when stored under these conditions (<0.06 % of HRP

per hematin), and resulting activity must therefore be attributed to the formation of holoHRP. The peroxidase activity at zero Mb is accounted for by the residual activity in the apoHRP preparation. It is evident that more HRP was formed at pH 10 than at pH 7. This could be anticipated since the transition of the acid, brown to the alkaline, red form of the ferrihemoproteins occurs with pK 8.9 for Mb [11,13] and 11 for HRP.[14] Hence Mb exists as Mb^+ at pH 7 and mainly as Mb·OH at pH 10, whereas HRP is in the HRP^+ form at pH 7 and mainly in this form at pH 10. Besides improving the accuracy of the spectrophotometric assays this circumstance would affect the equilibrium. Two to ten times higher concentrations of various nitrogenous ligands were required at pH 9.5 than at pH 7 for the half-formation of the heme-ligand complex,[9,15] and the hematin-protein association constants at 25° were found to be log K = 15.24 for Mb^+ and 12.50 for Mb·OH.[2] However, pH will also affect the free hematin ($pK_{H_2O,OH}$ = 7.6 [9]) and the apoproteins, and the influence of pH on the transfer of hematin obviously involves several factors.

Table 1. HRP and Mb after 24 h in a reaction mixture initially containing 57 μM apoHRP and 150 μM Mb. H_3BO_3-NaOH-KCl, pH 10.0, μ = 0.1. ε mM^{-1} cm^{-1} was found as 8.4 and 8.5 for Mb, and as 8.4 and 3.2 for HRP in this buffer at 532 and 585 mμ.

	Concentrations in μM according to			
	spectro-photo-metry	chro-mato-graphy	activity	Average
HRP	23.0	25.7	21.9	23.5
Mb	127.0	123.0		126.1
Sum	150.0	148.7		149.6

The results of the hematin transfer as measured by three methods are compared in Table 1. Equilibrium was possibly not fully attained, but the agreement between the results confirms that holoHRP was formed and that hematin did migrate from one protein to another.

Table 2. Determination of the ratio between the affinities of hematin for the protein moieties of Mb and HRP at pH 10. The table gives the concentrations of apoHRP and apoMb (0) before the addition of hematin (total concentration 50 μM) and the concentrations of HRP and Mb at 15 h (t). Buffer: H_3BO_3-NaOH-KCl, pH 10.0, μ = 0.1. The components were assayed by spectrophotometry (*cf.* Table 1) and peroxidase activity measurements. Concentrations in μM.

$$Q = \frac{(\text{apoMb}_0 - \text{Mb}_t) \times \text{HRP}_t}{\text{Mb}_t \times (\text{apoHRP}_0 - \text{HRP}_t)}$$

$apoMb_0$	$apoHRP_0$	Mb_t	HRP_t spectro-photometry	HRP_t act.detn.	Q
129	14	44	0.3	0.4	0.04
70	7.0	42	0.4	0.4	0.04
70	14	41	1.0	0.6	0.05
70	36	42	1.6	1.3	0.03
70	71	43	2.0	2.1	0.02
35	107	25	20	16	0.09
14	71	12	29	27	0.14
7.0	71	6.5	35	31	0.08
				Average	0.06

If the off-reaction is responsible for the slow attaining of the equilibrium it is expedient to add hematin to a mixture of the apoproteins.[2] From such experiments the ratio between the affinities of hematin for the protein moieties of Mb and HRP can be calculated, provided that the concentration of hematin is less than the sum of the concentrations of the binding sites. Under the conditions given in Table 2 hematin is apparently 17 times more firmly bound in Mb·OH than in HRP^+.

In neutral solutions the Soret bands of HRP and *Aplysia* Mb show similar relationships to the Soret band of horse Mb as regards height and position. This fact and our ratio between the association constants of Mb and HRP may be relevant to the absence of a measureable hematin transfer form horse Mb to *Aplysia* Mb.

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Effect of Ethionine on Spermidine, Spermine and Adenine Nucleotides in Rat Liver

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Experiments with chick embryos [1,2] demonstrated that ornithine, putrescine and methionine can act as precursors in the biosynthesis of the polyamines spermidine and spermine. Ethionine, a methionine analogue, decreased both the concentration of spermine and the incorporation of radioactivity into this polyamine after injection of labelled methionine,[2] effects which were attributed to the formation of S-adenosylethionine [3] with a decrease in adenosine triphosphate.[4,5] Ethionine treatment seems to afford information on the biosynthesis and metabolism of the polyamines. Present experiments deal with changes in the polyamines and adenine nucleotides in rat liver after administration of ethionine.

Methods. The animals used were female albino rats of the Wistar strain aged seven months and weighing 160 to 190 g, fed with a standard diet *ad libitum*. DL-Ethionine in 0.9 % NaCl was administered intraperitoneally as indicated in Table 1. For nucleotide determination liver samples were frozen *in situ* under ether anaesthesia,[6] excised, weighed and homogenized in perchloric acid with an Ultra-Turrax homogenizer. The acid extracts were neutralized and evaporated under a vacuum at 3–5°C. ADP, ATP and S–AE* were determined from the crude extracts by paper electrophoresis,[7] AMP after charcoal treatment.[8] Enzymic methods [9] for ADP and ATP gave parallel, though somewhat lower values. The polyamines were determined from the rest of the liver by paper electrophoresis.[2]

Results. Table 1 demonstrates the marked effect of ethionine on the liver polyamine contents. With the larger dose there was at first a significant decrease and then an increase in the spermidine concentration, whereas spermine decreased continuously. With the smaller dose the changes were similar, though not so marked or rapid. Calculation per organ reveals similar changes as per unit of wet weight.

As previously reported,[4,5,10] ethionine causes a decrease in the liver ADP and ATP, which is also seen in Fig. 1. With the larger dose ADP and ATP were at the level of 30–35 % of the control values at 24 h, but then increased, being near the controls 5 days later. A striking feature was the appearance of a UV-absorbing substance in the electropherograms from the ethionine-treated rats, not present in the control ones. By paper chromatography [11,12] this fraction was identified as S-adenosylethionine. The concentration of S–AE was highest, reaching almost the same value with both doses, at 24 h, after which it decreased rapidly with simultaneous increase in ADP and ATP.

Data obtained in isotope experiments with chick embryos indicate that the

* Abbreviations: AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; S–AE, S-adenosyl ethionine; S–AM, S-adenosylmethionine.