The alteration of the functional properties of human haemoglobin by spectrin

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Summary. Kinetic investigation by means of stopped flow techniques showed the rate of deoxygenation of haemoglobin to be slower in the presence of spectrin. At pH 7.15, the kinetic constant was 27.2 sec^{-1} in presence of spectrin instead of 34.3 sec^{-1} for haemoglobin alone. Also, equilibrium studies have revealed that the oxygen pressure for half-saturated haemoglobin decreased when spectrin was added to the reaction medium. At pH 7.35, the $\log (pO_2)_{1/2}$ was 0.88 for haemoglobin in presence of spectrin instead of 0.93 for haemoglobin alone. From these results, an interaction between spectrin and haemoglobin may be suspected.

Spectrin is a major component of the erythrocyte membranes. Purified spectrin gives raise to 2 major bands on sodium dodecyl-sulfate gel electrophoresis, corresponding to apparent mol. wt of about 220,000 and 200,000[3]. It may be considered as a 'peripheral' membrane protein, since it is easily extracted from membrane by means of low ionic strength aqueous solvents[4]. The location of spectrin at the inner surface of the red blood cell membrane with, as a consequence, a possibility of contact with haemoglobin, prompted us to investigate the effect of spectrin on the functional properties of the haemoprotein.

Material and methods. 1. Preparation of spectrin and haemoglobin. Human spectrin was extracted according to Marchesi[4]. It was further purified by means of gel filtration on sephadex G-200 using 25 mM Tris-HCl, 0.1 NaCl, 1 mM EDTA and 5 mM mercaptoethanol at pH 8.5 as the eluant. 650 mg of pure spectrin per liter of blood were routinely obtained. The purity of spectrin for each preparation, checked using SDS polyacrylamide gel[5], was found to be at least equivalent to those currently used in other laboratories.

Human haemoglobin was isolated after red cell lysis, according to well-known procedures[6]. Protein concentration were determined spectrophotometrically, using a Zeiss PMQ II spectrophotometer. For a 1% solution of spectrin, when measured at 280 nm, an absorbance value of 10.7 was used[7]. For oxyhaemoglobin, the molar extinction coefficient at 540 nm was taken as $1.37 \times 10^{+4}$ $M^{-1}\,cm^{-1}$ (Antonini[8]).

2. Binding of oxygen to haemoglobin: tonometric measurements. Spectrophotometric measurements at both 560 and 575 nm, using either a Cary 15 M or a Zeiss PMQ II spectrophotometer, allowed us to determine the affinity of haemoglobin for oxygen according to Rossi et al.[9]. Before each experiment, haemoglobin and spectrin solutions were dialyzed for 4 h at 4 °C against 0.1 Tris-HCl buffer at pH 7.3, containing 10 mM KCl, 10 mM $MgCl_2$ and 5 mM mercaptoethanol. The protein concentrations were adjusted to 1.3×10^{-5} M for haemoglobin and 7.7×10^{-6} M for spectrin, respectively.

3. Deoxygenation of haemoglobin: kinetic measurements. The kinetics of deoxygenation of haemoglobin were studied by means of the stopped flow technique. The mechanical module of the stopped flow apparatus was obtained by courtesy of Prof. H. Gutfreund; it is characterized by a dead time of 3 msec. The split beam, differential spectrophotometer is equipped with a Foci monochromator. 2 EMI 9601B photomultipliers are used for the observation beam and for the reference beam respectively. Their differential potential, which is proportional to the difference in absorbance between the 2 beams, is stored by means of a Datalab transient recorder (type DL 905). The resulting time curves are displayed either on a Tektronix oscilloscope (type D 13) or on a XY Bryans recorder (type 29000 A4).

The deoxygenation process was started by rapidly mixing oxyhaemoglobin with a 0.2% sodium dithionite solution prepared in deoxygenated buffer[10]. All solutions were prepared like those used in equilibrium experiments. Kinetic runs were carried out at 25 °C, the deoxygenation reaction being followed by observing the change in absorbance at 475 nm.

Results and discussion. A. Equilibrium studies. In table 1 are listed the Hill's coefficient (n) and the logarithm of oxygen partial pressure at half-saturation ($\log (pO_2)_{1/2}$) characterizing the interaction of oxygen with haemoglobin. When the values of Hill's coefficient in the absence and in the presence of spectrin are compared, the hypothesis that haemoglobin dissociates from tetramer to dimer under the influence of spectrin can be ruled out. Indeed, Hill's coefficient clearly suggests that the haem-haem interactions remain practically unaffected by the presence of spectrin. If, on the other hand, the oxygen pressure at half-saturation is considered, each individual experiment has revealed a small but significant decrease in the

Table 1. Tonometric investigation of the equilibrium between haemoglobin and oxygen

	Haemoglobin alone (1.3×10^{-5} M)	Haemoglobin (1.3×10^{-5} M) plus spectrin (7.7×10^{-6} M)
n	2.97 ± 0.2	2.90 ± 0.3
$(\log (pO_2)_{1/2})$	0.93 ± 0.02	0.88 ± 0.02

For experimental conditions, see text. Each value is the average of 10 separate experiments. ± SD.

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Table 2. Kinetic investigation of the release of oxygen from haemoglobin

	(A) Haemoglobin (2–8 μM)	(B) Haemoglobin plus spectrin	(B)/(A)	Haemoglobin plus lysozyme
pH = 6.2 (unbuffered)	47.9 sec^{-1}	37.5 sec^{-1}	0.78	–
pH 7.15 (0.1 M phosphate)	34.3 sec^{-1}	27.2 sec^{-1}	0.79	34.7 sec^{-1}
pH 7.3 (0.1 M Tris/HCl)	31.5 sec^{-1}	27.7 sec^{-1}	0.88	31.5 sec^{-1}

The results are calculated as first-order velocity constants, each value is the average of 5 separate experiments; the standard deviation is close to 1 sec^{-1} in each case. For experimental conditions, see text. The molar ratio of spectrin to haemoglobin was 0.3 at pH 6.2, 1.1 at pH 7.15 and 0.9 at pH 7.3; the molar ratio of lysozyme to haemoglobin was 0.9.

value of $\log (pO_2)_{1/2}$ when spectrin was present. Therefore it appears that, at any given oxygen pressure, a slightly greater proportion of oxyhaemoglobin is formed as a result of the presence of spectrin.

B. Kinetic investigations. Comparative measurements have been carried out in which haemoglobin was deoxygenated either alone, or in the presence of spectrin, or another protein, lysozyme. The measurements were repeated at different pH values in the range 6–7.5. In our experimental conditions, the kinetics were pseudofirst order. (See also Salhany et al.[11].)

From inspection of table 2, it will be apparent that, at any of the 3 pH investigated, the presence of spectrin slows down the speed of the deoxygenation process of haemoglobin (while another protein, lysozyme, does not). In conclusion, kinetic as well as equilibrium studies lead one to admit that the presence of spectrin favours, to a measurable extent, the oxygenated form of haemoglobin. These experimental results suggest that an interaction between spectrin and haemoglobin can be awaited. Such an interaction is now being investigated in our laboratory.

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Central tyramine prevents hypertension in uninephrectomized DOCA-saline treated rats

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Summary. Prevention of high blood pressure in uninephrectomized, DOCA-saline treated rats was observed after treatment with central tyramine precursors. We suggest that the high blood pressure is either due to relative lack of tyrosine, which might be caused by the hyperactivity of tyrosine hydroxylase, or to hypoactivity of the decarboxylase: in both cases the result is diminished tyramine synthesis.

The rate-limiting step of catecholamine synthesis is the formation of L-DOPA from tyrosine by tyrosine hydroxylase (TH). The best known inhibitors for TH are alpha-methyl tyrosine[3], Pyratrione[4] and Oudenon[5]. All these agents decrease or prevent high blood pressure. The formation of noradrenaline from dopamine is mediated by dopamine beta hydroxylase (DBH). Inhibitors of this enzyme, such as Dopastine[5], disulfiram (Antabuse)[6] and fusaric acid[7], also decrease or prevent high blood pressure. Destruction of central adrenergic neurons by intraventricular 6-hydroxydopamine prevents the induction of hypertension[8]. Furthermore, Nagatsu et al.[9] found that NaCl administration to SH rats caused an increase in TH activity in several organs in addition to the congenitally elevated hypothalamic one[10]. Rylett et al.[11] showed that treatment with DOCA increased tyrosine hydroxylase activity and therefore increased the amount of tyrosine which enters the pathway of noradrenaline synthesis. One might therefore conclude that the inhibition of noradrenaline synthesis is responsible for the antihypertensive effect. Paradoxically, however, Lavorit and Valette[12] found that administration of tyrosine to DOCA-saline treated rats also prevents the elevation of blood pressure and speculated on the importance of central noradrenergic hypotensive centres. To eliminate the inconsistencies we hypothesized that preventing the elevation of blood pressure is due to a tyrosine metabolite not in the pathway of catecholamines' synthesis, since excess dietary tyrosine in rats does not change the catecholamine concentration in the brain[13], but still decrease the blood pressure.

Materials and methods. In this work, uninephrectomized male rats weighing 180–200 g were used. Desoxycorticosterone acetate (DOCA) 10 mg/kg was injected 3 times a week s.c. Food and saline (0.9% NaCl) were given ad libitum. Blood pressure was measured under light ether anaesthesia by a tail microphonic method[14]. For statistical analysis the Student t-test was used.

Results and discussion. From table 1 we can see that L-tyrosine 500 mg/kg totally prevents the elevation of blood pressure. The same is true for α-methyltyrosine, an inhibitor of tyrosine hydroxylase. Table 2 shows that L-tyrosine is also able to diminish existing high blood pressure. The experimental data in table 1 point to tyramine as the central hypotensive agent. This is suggested by the tyramine experiment as well as by the synergism of an ineffective dose of tyrosine with Vit. B_6 – the cofactor of amino acid decarboxylase, and the synergism of the same ineffective dose with carbidopa – a peripheral decarboxylase inhibitor. The final proof is, however, the high efficiency of D, L-tyrosine as compared with L-tyrosine[28] explained by the fact that TH is specific for the L form, but deamination shows no such specificity[15]. Finally, neither D nor L phenylalanine had any effect, indicating lack of effect of phenylethylamine. L-tryptophan is also ineffective.

Let us see how other observations fit our hypothesis. Raese et al.[16] showed that presynaptic tyrosine hydroxylase is activated by c-AMP. Dopamine inhibits that activation, therefore elevation of dopamine level, for example, by giving a precursor or inhibiting its metabolism, will inhibit tyrosine hydroxylase. DOPA indeed is