A molecular mechanism for the biphasic effect of exogenous arachidonate on platelets

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A haemoprotein ($M_r \sim 40\,000$) made up of two apparently identical subunits, and having Soret maximum at 405 nm, but displaying it mostly at about 410 nm or often at 415 nm in crude extracts owing to different bound small molecules, found in platelets of several species, has been purified from calf platelets. The purified protein could bind the stable PGH_2 analogue, U-46619, without co-operativity ($h = 1.0$ and half-maximal saturation concentration, $S_{0.5} = 10\ \mu M$) and arachidonate with co-operativity that increased with arachidonate concentration (h increasing from ~ 2 to ~ 4). $S_{0.5}$ of arachidonate was 1.5 μM. Arachidonate binding to the protein was accompanied by its oligomerization.

Platelet *Hemoprotein* *Arachidonate* *Receptor* *U-46619* *Co-operativity* *(Calf)*

1. INTRODUCTION

Exogenous arachidonic acid (5,8,11,14-eicosatetraenoic acid) aggregates blood platelets of a number of species but the response follows a bell-shaped pattern, higher concentrations becoming progressively more inhibitory to the aggregation by lower concentrations [1–4]. Inhibition takes effect apparently without damage to platelets and without impairment of arachidonic acid metabolism [5] but without having recourse to metabolic conversion of arachidonic acid [2,3,5,6] although 12-hydroperoxyeicosatetraenoic acid, a lipoxygenase product, is an inhibitor [4]. The biochemical mechanism of the direct inhibitory effect is not clear but the recent observation of Huang and Detwiler [6] that arachidonic acid directly antagonized platelet activation by the stable synthetic prostaglandin endoperoxide analogue, (15*S*)-hydroxy-11α,9α-epoxymethano-5*Z*,13*E*-dienoic acid (U-46619), raised the possibility of involvement of an endoperoxide receptor. Since peroxide-binding proteins are usually haemoproteins we looked for a haemoprotein, distinct from the arachidonate cyclo-oxygenase, which could bind arachidonate as well as U-46619 in platelets. Here we describe for the first time, the purification of such a haemoprotein from calf platelets.

2. MATERIALS AND METHODS

2.1. *Materials*

Sephadex gels were from Pharmacia, Sweden. Arachidonic acid, a product of Sigma, was dissolved in absolute alcohol and its concentration estimated spectrophotometrically using E_M^{270} 4750 $M^{-1} \cdot cm^{-1}$ [7]. U-46619, a product of Upjohn, Kalamazoo, MI, obtained through the courtesy of Dr J. Lakshmanan, of the University of California, Torrance, was dissolved in absolute ethanol and its concentration in experimental systems was estimated using E_M^{235} 10000 $M^{-1} \cdot cm^{-1}$ in alcohol, arbitrarily, for the sake of consistency in different experimental situations.

2.2. *Preparation of platelets*

Calf (male) blood (100 ml) was collected by jugular venic puncture into acid-citrate-dextrose solution (15 ml) and the platelets were purified by

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an adaptation of the method described by Thompson et al. [8]. The concentration of bovine serum albumin was reduced to one tenth that used by them. Platelet-rich plasma was prepared by differential centrifugation at 32°C and 100 × g ($r_{av.}$ 10 cm) for 1 h. Platelets were pelleted from the platelet-rich plasma by centrifugation at 32°C and 450 × g ($r_{av.}$ 10 cm), washed twice in the buffer system of Thompson et al. [8], modified as above, and resuspended in 100 ml of the non-colloidal buffer system of Tangen et al. [9].

2.3. *Purification of platelet haemoprotein*

Purified platelets (20 ml) were broken ultrasonically at 20°C and centrifuged for 30 min at 4°C and 12000 × g ($r_{av.}$ 8 cm) to obtain a crude extract. The crude extract (110 ml, A_{405} = 2.3) was precipitated with $(NH_4)_2SO_4$ at 4°C and the second fraction, precipitating between 0.5–0.9 saturation was collected by centrifugation at 12000 × g for 30 min and dissolved in 2 ml of 0.05 M sodium phosphate buffer (pH 7.4) (referred to hereafter as the buffer) and clarified by centrifugation. The clear supernatant (A_{405} ~ 1.1) was applied to a column of Sephadex G-200 (1.2 × 50.5 cm) equilibrated with the buffer at 4°C. The column was eluted at a flow rate of 13 ml·h^{-1} and haemoprotein fractions, followed by absorbance at 405 nm, eluting between 31–50 ml were pooled (A_{405} ~ 0.8) and precipitated with solid $(NH_4)_2SO_4$ added to 0.95 saturation at 4°C. The protein precipitated overnight was collected by centrifugation and dissolved in 1 ml of the buffer, and dialyzed against the same buffer for 6 h at 4°C. The dialysis residue was applied to a column (1.1 × 28.5 cm) of DEAE Sephadex A-50 equilibrated and eluted with the buffer at 4°C and at a flow rate of 13 ml·h^{-1}. The haemoprotein fractions (15–24 ml) were pooled, precipitated with $(NH_4)_2SO_4$ dissolved in 1 ml, dialyzed against the buffer, and used for the studies reported here.

3. RESULTS

The purification procedure described here resulted in a preparation (1 ml, A_{405} = 0.12) having 75 μg protein compared to the 12000 × g supernatant (110 ml, A_{405} = 2.3) containing 46 mg of protein estimated by the method of Lowry et al. [10]. The elution volume of the haemoprotein from the column of Sephadex G-200 when compared to those of globular proteins of known M_r corresponded to an M_r of 40000. SDS-polyacrylamide gel electrophoresis (PAGE) gave a major band at M_r = 21000 (fig.1). The same subunit M_r was obtained under nonreducing conditions also showing that two apparently identical subunits were held together by noncovalent forces. Minor bands were also seen at M_r = 42000 and ~82000 in the gel.

The purified protein showed Soret maximum at 405 nm (E_{mM} ~ 65 mM^{-1}·cm^{-1}) which upon reduction with $Na_2S_2O_4$ shifted to 424 nm (E_{mM} ~ 40 mM^{-1}·cm^{-1}) suggesting the haem iron to be in the +3 oxidation state.

Treatment of the protein with arachidonate shifted its Soret band to 410 nm (fig.2A). The

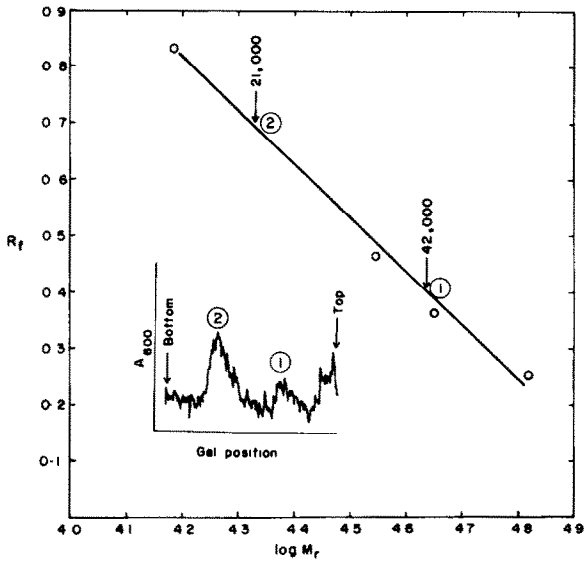

Fig.1. SDS-PAGE pattern of purified calf platelet haemoprotein. The protein (140 μg) in 2 ml of 0.05 M sodium phosphate buffer (pH 7.4), was concentrated nearly to dryness by dialysis against solid Sephadex G-25. It was then dialyzed against distilled water for 1 h and the dialysis residue was taken up in the sample buffer for electrophoresis in 10% cylindrical gels according to the Sigma Technical Bulletin MWS-877 at 32°C with M_r marker proteins in separate gels. The destained gel was scanned at 600 nm in a Shimadzu UV-240 spectrophotometer using its gel-scanner attachment. The marker proteins employed (with their log M_r values in parentheses) were: lysozyme (4.16), pepsin (4.54), ovalbumin (4.65), and bovine serum albumin (4.82).

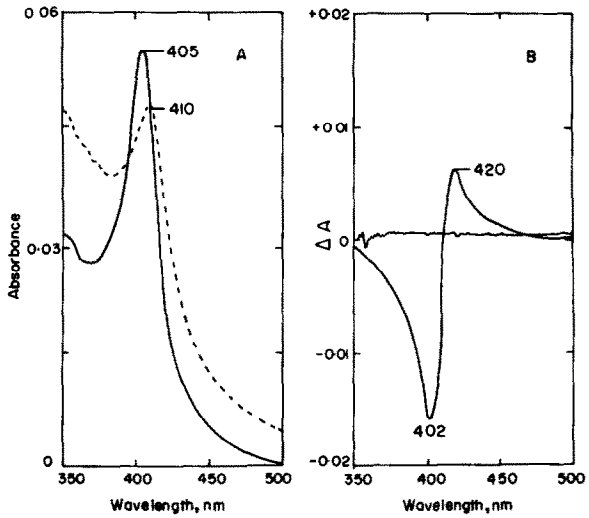

Fig.2. (A) Effect of arachidonic acid on the aerobic electronic absorption spectrum of platelet haemoprotein. Purified calf platelet haemoprotein, in 0.05 M sodium phosphate buffer (pH 7.4), having the solid line spectrum was treated with a 1 μl sample of arachidonic acid in ethanol and the spectrum recorded again (broken line), at 35°C. The final arachidonic acid concentration was 8.4 μM. (B) A difference absorption spectrum obtained after adding arachidonic acid (7.5 μM) to the purified platelet haemoprotein in 0.05 M sodium phosphate buffer (pH 7.4) at 35°C. Protein sample (1 ml, A_{405} = 0.07) was taken in each of a pair of matched spectrophotometric cuvettes (pathlength 10 mm) and the base-line was corrected automatically, and recorded. A 1 μl sample of arachidonic acid in ethanol was added to the sample cuvette while 1 μl ethanol was added to the reference cuvette. The spectrum was recorded 5 min after mixing.

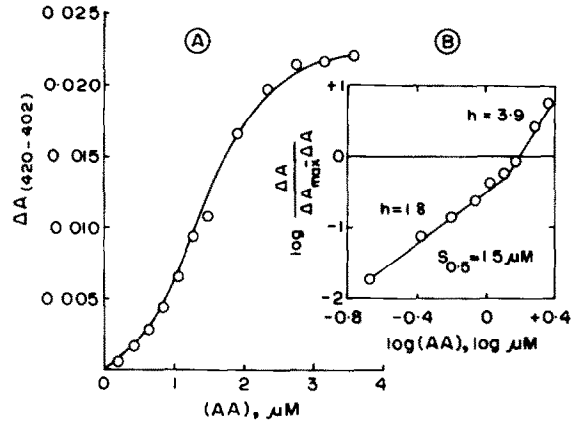

Fig.3. (A) Variation of $\Delta A_{(420-402)}$ as a function of arachidonate concentration. (B) Hill plot of the data. Experimental conditions were those of fig.2B except for the use of a freshly prepared solution of arachidonic acid in 100 mM Na_2CO_3, the variation in arachidonate concentration and the employment of a lower temperature, 32°C. The total volume of arachidonate solution added did not exceed 10 μl.

shifted band remained quite stable. A difference spectrum of the arachidonate-treated protein against the untreated protein showed a peak at 420 nm and a trough near 402 nm (fig.2B). Gel filtration of the arachidonate-treated protein, in a column of Sephadex G-25, reverted the Soret band back to 405 nm (not shown). These results showed reversible binding of arachidonate to the protein. Neither the solvent nor a saturated fatty acid, stearic acid (0.8 μM), could produce a spectral effect, but another unsaturated fatty acid, homo-γ-linolenate (8,11,14-eicosatrienoate, Sigma), yet another, apparently direct, inhibitor of U-46619-induced platelet aggregation [6], did show spectral effects similar to arachidonate (not shown).

The magnitude of the difference spectral change induced by arachidonate was dependent on the concentration of arachidonate (fig.3A) and a Hill plot showed two linear regions with Hill coefficient, h ~2 at low arachidonate concentrations, increasing to ~4 at higher concentrations (fig.3B). Thus the degree of positive co-operativity ($h > 1$) of arachidonate binding to the protein increased as the arachidonate concentration increased. The concentration of arachidonate at half-maximal saturation was 1.5 μM under the test conditions.

An $h > 2$ was unexpected for a dimeric protein. But it was found, in a large-zone gel exclusion chromatographic experiment, that treatment of the protein (1 ml, A_{405} = 0.05) with arachidonate (7.5 μM) reduced its elution volume, from 4.8 to 4 ml, in a Sephadex G-200 column (0.6 × 17 cm), at 5°C. This arachidonate-induced oligomerization of the protein, could account for the high degree of co-operativity.

Difference spectra obtained on adding U-46619 to the purified protein also showed concentration-dependent binding interactions (fig.4). The spectra did not reveal any distinct peak but a hump at about 428 nm seen at high U-46619 concentrations. The trough was at 405 nm and a plot of $(\Delta A_{405})^{-1}$ against $(\text{U-46619})^{-1}$ was linear (fig.5). A

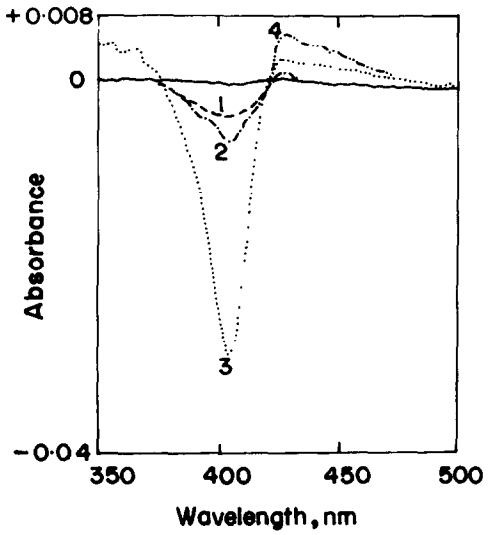

Fig.4. Difference spectra obtained on adding different concentrations of U-46619 to purified platelet haemoprotein. An alcoholic solution of U-46619 was mixed with the haemoprotein in 0.05 M sodium phosphate buffer (pH 7.4) (1 ml, A_{405} = 0.087) at final U-46619 concentrations of 3.3 μM (curve 1), 14.8 μM (curve 2) and 33 μM (curve 3) and the difference spectra were recorded at 32°C as described under fig.2B, except for the use of U-46619 solution, the total volume of which did not exceed 15 μl. Curve 4 was the positive part of curve 3 recorded at 2 × expanded scale to show the position of the hump.

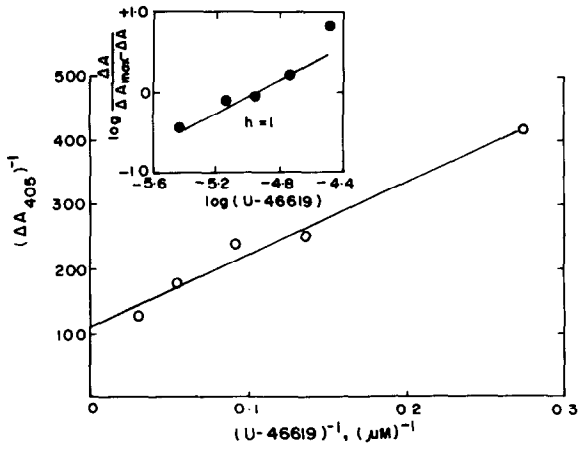

Fig.5. Double reciprocal plot and Hill plot (inset) of a set of data relating the magnitude of spectral change obtained at 405 nm (ΔA_{405}) at various U-46619 concentrations. Experimental conditions were those of fig.4.

Hill plot of the data was also linear (fig.5, inset) with h equal to unity, suggesting that the binding interaction was devoid of co-operativity. Half-maximal saturation concentration of U-46619 was 10 μM.

4. DISCUSSION

The SDS-PAGE pattern of the purified haemoprotein showed a major band corresponding to M_r = 21000. But it also showed minor bands at M_r = 42000 and ~82000. Since the M_r of the pooled fractions from the Sephadex G-200 column could not have exceeded ~50000 and since nondenaturing PAGE of the purified protein at pH 7.0 and 8.3 showed only one band (not shown), we conclude that the high M_r material was derived from aggregates formed during its dialysis against solid Sephadex to concentrate it for electrophoresis. This treatment was found to result in haem loss and aggregation of the apoprotein. The 42-kDa and ~82-kDa bands could then be derived from the dissociation of the aggregated material, during SDS treatment. The appearance of the bands at M_r positions which were integral multiples of the subunit molecular mass made this a reasonable possibility. But the possible presence of a 42-kDa contaminant or associated protein (actin!) is not ruled out.

The platelet preparations used for the purification of the protein had been carefully freed from other cell types and adsorbed proteins. So the protein originated from platelets. Its soluble nature and molecular mass (40 kDa) clearly demarcate the protein from the other well-known platelet haemoprotein, the arachidonate cyclo-oxygenase [11,12] which is particulate and has a subunit molecular mass (~79–85 kDa) vastly in excess of that found here [13–15]. Further, although addition of arachidonate to the purified cyclo-oxygenase produced a spectral shift (409 to 412 nm), the stability of the shifted Soret band observed here was in sharp contrast to its rapid destruction in the case of the cyclo-oxygenase [12]. But the oxidation state of the haem iron and the millimolar absorbance of the Soret bands of the two proteins seem to be similar [11] and the two proteins lose their haem prosthetic group quite easily.

The ability of the protein to bind U-46619 made it a likely candidate for being the proaggregatory endoperoxide receptor. The following results provided additional support to this conclusion. PGG_2 or PGH_2, prepared by the method of Hamberg et al. [16], on adding to the protein, yielded difference spectra having peaks at 425 or at about 427 nm (in the case of PGH_2) and troughs at 405 nm, suggesting their binding to the protein. Prior treatment of these labile compounds with water or methanol, known to destroy the endoperoxide group, abolished their ability to bring about the characteristic spectral change. This result suggested that it was the intact endoperoxide molecules themselves, rather than some of their decomposition products, that were responsible for the binding. The binding of U-46619 to the protein was found, by circular dichroism measurements, to result in a dose-dependent decrease in the α-helical content of the protein (unpublished), showing that it did bring about a conformational change although there was no suggestion of co-operativity in the Hill plot. It was found, further, that imidazole, another haem ligand, could induce positive co-operativity in the binding of U-46619 to the protein and hinder the binding of arachidonate to it by noncompetitive type of mechanisms; and true to expectation, imidazole enhanced the rates of aggregation of gel-filtered calf platelets by U-46619 (unpublished). And 8,11,14-eicosatrienoate which, like arachidonate, is a direct inhibitor of U-46619-induced platelet aggregation [6], did indeed bind to the protein while stearic acid, not known to affect platelet aggregation, did not.

We envisage the haemoprotein receptor, which we have found in the platelets of such widely different species as calf, sheep and human, to be positioned somewhere near the cytoplasmic side of the plasma membrane of the platelet dense tubular system where the cyclo-oxygenase is located [17,18]. The cyclo-oxygenase products, PGG_2 and PGH_2, have free access to the receptor as well as to the membranous thromboxane A_2 synthetase, possibly also a haemoprotein [19], whose half-maximal saturation concentration is, apparently, higher than that of the endoperoxide receptor. The receptor, upon interaction with the endoperoxides, undergoes a conformational change, triggering the platelet activation cascade. In case there is a surfeit of arachidonate, or an analogous unsaturated fatty acid, it could overflow directly to the endoperoxide receptor, resulting in its allosteric alteration and making it less amenable to the proaggregatory endoperoxides. This is, apparently, the molecular mechanism of the direct antiaggregatory effect of exogenous arachidonate, and some other unsaturated fatty acids, on platelets.

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