Kinetics of inhibition of sperm β-acrosin activity by suramin

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Abstract Sperm β-acrosin activity is inhibited by suramin, a polysulfonated naphthylurea compound with therapeutic potential as a combined antifertility agent and microbicide. A kinetic analysis of enzyme inhibition suggests that three and four molecules of suramin bind to one molecule of ram and boar β-acrosins respectively. Surface charge distribution models of boar β-acrosin based on its crystal structure indicate several positively charged exosites that represent potential 'docking' regions for suramin. It is hypothesised that the spatial arrangement and distance between these exosites determines the capacity of β-acrosin to bind suramin.

Key words: Spermatozoa; β-Acrosin; Fertilisation; Suramin; Serine protease; Contraception

1. Introduction

Proacrosin, the zymogen form of the serine protease β-acrosin (EC 3.4.21.10), is found specifically within the acrosomal vesicle of spermatozoa from ascidians to mammals [1–3]. Its primary sequence shows significant identity to other serine proteases such as trypsin (35%), chymotrypsin (33%), elastase (29%) and kallikrein (27%) [1]. Proacrosin is also one of a cohort of reproductive proteins that show a high rate of sequence diversification, a characteristic feature of adaptive evolutionary processes that contribute to speciation [4]. In intact spermatozoa proacrosin is complexed to several binding proteins and inhibitors within the acrosomal matrix and is only converted to β-acrosin following exocytosis of the acrosomal vesicle. Unusually for a serine protease, it autoactivates by internal cleavages at both the N- and C-termini to produce a truncated two-chain molecule cross-linked by disulfide bridges [5]. Recently, the three-dimensional structures of boar and ram sperm β-acrosins were determined by X-ray crystallography and were noteworthy for the presence of positively charged 'patches' of basic residues close to the active site [6]. These patches allowed close apposition of adjacent molecules within the crystal stack via ionic bonding of charged carbohydrate chains. From a physiological standpoint, β-acrosin is thought to be a multifunctional protein with putative roles during fertilisation as a secondary zona binding molecule [7,8], for facilitating dispersal of the acrosomal matrix [9] and as an activator of protease activated receptor 2 (PAR2) on the oolemma following zona penetration [10].

In previous experiments we reported that the drug suramin inhibits both the amidase activity of β-acrosin and binding of spermatozoa to the zona pellucida (ZP) in vitro [11]. Suramin is a symmetrical hexasulfonated naphthylurea compound (Fig. 1) that was originally synthesised as a trypanocidal agent for treatment of sleeping sickness in East Africa [12]. Lately, it has found diverse applications as an anticancer drug because of its ability to block angiogenesis, inhibit reverse transcriptase and cause microaggregation of growth factors [13,14]. Although suramin binds to a variety of proteins (e.g. human chymase [15], protein tyrosine phosphatase [16] and heparinase [17]) it can also discriminate between closely related members of the same family suggesting that the 'docking' sites are very precise. Thus, it is a potent inhibitor of human elastase, neutrophil protease 3 and cathepsin G but has little or no effect on pancreatic trypsin and chymotrypsin [18]. Binding of suramin is principally ionic and depends on the correct spatial alignment between sulfonate groups on the terminal naphthalene rings and basic residues on the surface of the target protein in a manner similar to that found for heparin–antithrombin III interactions [19]. The ratio of suramin binding to protein is often greater than 1, suggesting the presence of several docking sites [18,20].

The inhibitory effects of suramin on secondary binding of spermatozoa to the ZP of the egg [8], together with its antiviral activity [21], suggest that it has potential as a combined antifertility agent and microbicide. To further substantiate this hypothesis, we have investigated the stoichiometry and kinetics of binding of suramin to β-acrosin from ram and boar spermatozoa. We show that either three or four molecules of suramin bind per molecule of β-acrosin and predict potential binding sites on the surface of the protein from charge density models of its three-dimensional structure.

2. Materials and methods

2.1. Materials

Substrate S2288 (D-Ile-Pro-Arg-*p*-nitroanilide) was purchased from Helena Laboratories (Mt. Waverly, Australia). *p*-Nitrophenyl *p*′-guanidino-benzoate, methyl-umbelliferyl-guanidino-benzoate and methyl-umbelliferone were obtained from Sigma (Sydney, Australia). Suramin hexasodium salt was supplied by AG Scientific (San Diego, CA, USA). β-Acrosin was purified to homogeneity from ejaculated ram and boar spermatozoa as described previously [22]. The protein gave a single band at ~38 kDa following sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)/Coomassie blue staining and was judged to be >95% pure by densitometry. The protein

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Abbreviations: ZP, zona pellucida

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was lyophilised and stored at −20°C. Under these conditions the enzyme is stable for several years.

2.2. Enzyme assays and kinetic analysis

Boar β-acrosin was titrated with *p*-nitrophenyl *p'*-guanidino-benzoate in 0.035 M sodium barbitone pH 8.3 at 25°C [23]. Ram β-acrosin was titrated with methyl-umbelliferyl-guanidino-benzoate in 0.035 M sodium barbitone pH 8.3 at 37°C using a Perkin Elmer luminescence spectrometer LS50B (excitation, 365 nm; emission, 445 nm) [24].

Chromogenic assays were performed at 37°C in 40 mM HEPES, 20 mM $CaCl_2$, 200 mM NaCl, 0.1% polyethylene glycol (PEG) 6000, 1 mg/ml bovine serum albumin (BSA), pH 7.4 at 405 nm using a Hewlett Packard 8452A diode array spectrophotometer. Before use plastic 1.5 ml cuvettes were coated with a solution of 0.1 M $NaHCO_3$, 1% casein, 0.02% sodium azide to prevent non-specific adsorption of proteins in the reaction mixtures to the cuvettes. Inhibition studies were only performed with batches of β-acrosin for which the rate of hydrolysis of S2288 demonstrated a linear dependence on enzyme concentration. The S2288 concentration was determined from its A_{342} using a molar absorption coefficient of 8270 M^{-1} cm^{-1} [25].

Initial rates for the hydrolysis of S2288 by ram and boar β-acrosins were measured using a range of S2288 and suramin concentrations. At least six concentrations of S2288 were tested, with at least one below the K_m^{app} and one above the K_m^{app}. For each suramin concentration, the data were fitted to the Michaelis–Menten equation (Eq. 1) by non-linear regression:

$$v_0 = \frac{[E]_0 k_{cat} k_A}{k_{cat} + k_A[A]} \quad (1)$$

where k_{cat} is the catalytic rate constant and k_A is the specificity constant k_{cat}/K_m.

Non-linear regression was used to analyse the dependence of the apparent kinetic parameters with inhibitor concentration. When Eqs. 2, 3 and 4 were fitted the value of k_{cat} or k_A was fixed. For ram β-acrosin $k_{cat} = 31 \pm 1$ s^{-1} and $k_A = (2.2 \pm 0.1) \times 10^5$ M^{-1} s^{-1} and for boar β-acrosin $k_{cat} = 3.2 \pm 0.1$ s^{-1} and $k_A = (5.4 \pm 0.6) \times 10^5$ M^{-1} s^{-1}.

3. Results and discussion

3.1. Kinetic parameters of suramin binding on the activities of ram and boar β-acrosins

For each suramin concentration we obtained apparent values of the specificity constant and the catalytic rate constant. Pure competitive inhibitors affect only the specificity constant whereas uncompetitive inhibitors affect only the catalytic rate constant. Secondary plots of the inverse of k_A^{app} and k_{cat}^{app} against inhibitor concentration are linear if the enzyme is completely inhibited by one inhibitor molecule [26]. Complete linear inhibition was not observed for the inhibition of β-acrosin by suramin (Fig. 1), so the data were fitted to equations that describe incomplete inhibition by more than one inhibitor molecule.

Surprisingly, the pattern of inhibition by suramin was different for ram and boar β-acrosins. For ram β-acrosin, no competitive inhibition was observed. In fact, a single suramin molecule actually increased the specificity of ram β-acrosin for the substrate (Fig. 2a). The data for the activation of ram

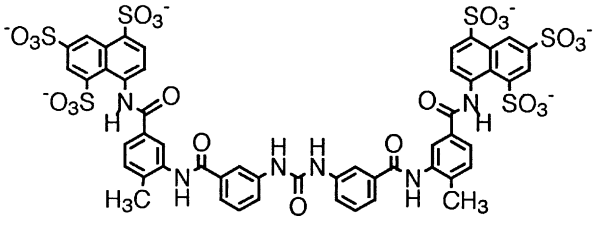

Fig. 1. Structural formula of suramin.

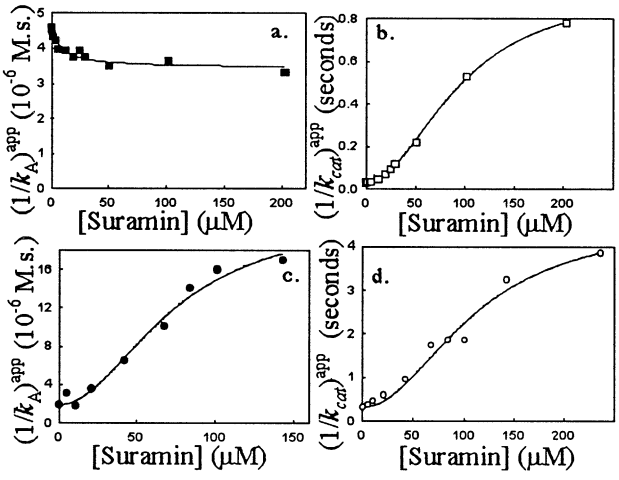

Fig. 2. The effect of suramin on $(1/k_A)^{app}$ (a and c) and on $(1/k_{cat})^{app}$ (b and d) for ram β-acrosin (a and b) and boar β-acrosin (c and d).

β-acrosin by suramin were fitted by Eq. 2 which describes the increase of the specificity constant by a single suramin molecule [26].

$$\frac{1}{k_A^{app}} = \left(\frac{1}{k_A}\right)\frac{1 + \frac{[I]}{K_{act}}}{1 + b\frac{[I]}{K_{act}}} \quad (2)$$

where K_{act} is the equilibrium dissociation constant for the activating suramin molecule and $b = k_A^{app}/k_A$ when [suramin] = ∞.

In contrast to ram β-acrosin, competitive inhibition was observed for boar β-acrosin (Fig. 2c). Despite repeating the data collection a number of times, the effect of suramin on the k_A^{app} for boar β-acrosin had a high degree of error within it. However, it is clear that partial (90%) competitive inhibition by more than one suramin molecule is occurring. The data were fitted by Eq. 3, which describes cooperative inhibition by two inhibitor molecules as shown in Scheme 1 [26].

$$\frac{1}{k_A^{app}} = \left(\frac{1}{k_A}\right)\frac{1 + \frac{[I]^2}{K_{ic}^2}}{1 + b\frac{[I]^2}{K_{ic}^2}} \quad (3)$$

where $b = k_A^{app}/k_A$ when [suramin] = ∞ and K_{ic}^2 is the dissociation constant for Scheme 1 for the competitive inhibitory suramin molecules.

For both boar and ram β-acrosins, the effect of suramin on the k_{cat}^{app} was similar (Fig. 2b,d); partial inhibition by more than one suramin molecule was observed. Different equations that describe two inhibitor molecules, two inhibitor molecules binding cooperatively and three inhibitor molecules were all used to fit the data. Eq. 4 fitted the data best, and this describes cooperative inhibition by two inhibitor molecules as shown in Scheme 1. The values for the parameters obtained by fitting the equations are shown in Table 1.

$$E + 2I \rightleftharpoons EI_2$$

Scheme 1.

Table 1
Kinetic parameters of inhibition of activity of ram and boar β-acrosins by suramin

	Effect on k_A^{app}	Effect on k_{cat}^{app}
Ram β-acrosin	$K_{act} = 10 \pm 3$ μM $b = 1.33 \pm 0.03$	$K_{iu} = 17.3 \pm 0.2$ μM $b = 0.0335 \pm 0.0004$
Boar β-acrosin	$K_{ic} = 22 \pm 2$ μM $b = 0.086 \pm 0.006$	$K_{iu} = 27 \pm 1$ μM $b = 0.070 \pm 0.003$

K_{act} is the equilibrium dissociation constant for the activating suramin molecule. K_{iu}^2 and K_{ic}^2 are the equilibrium dissociation constants for Scheme 1 for the uncompetitive and competitive inhibitory suramin molecules, respectively. b is the fraction of either k_A or k_{cat} that exists at infinite suramin concentration.

$$\frac{1}{k_{cat}^{app}} = \left(\frac{1}{k_{cat}}\right)\frac{1+\frac{[I]^2}{K_{iu}^2}}{1+b\frac{[I]^2}{K_{iu}^2}} \quad (4)$$

where $b = k_{cat}^{app}/k_{cat}$ when [suramin] = ∞ and K_{iu}^2 is the dissociation constant for Scheme 1 for the uncompetitive inhibitory suramin molecules.

3.2. Charge density models of suramin binding sites on boar β-acrosin

The three-dimensional structure of ram and boar β-acrosins is known from X-ray crystallography and hence it is possible to model the distribution of potential anion binding exosites using GRASP computer programmes. As shown in Fig. 3, the catalytic centre of boar β-acrosin, consisting of His70+Asp124+Ser222, is surrounded by two major anion binding regions represented by His47+Arg50+Arg51 and Arg250+Lys252+Arg253. (Note: the numbering system adopted here follows that of Baba et al. [5] for boar proacrosin which begins with the N-terminal residue (Arg) of the light chain. This is different to the chymotrypsin numbering system used by Tranter et al. [6].) These residues have been shown by site-directed mutagenesis to be important for ZP binding (and by inference suramin binding) whereas an adjacent area containing three lysines (Lys75+Lys76+Lys77) does not appear to be crucial, possibly due to the position of the latter in a 'pocket' formed by projecting surface loops [27]. Rotation of the protein about the *Y*-axis reveals several additional exosites that, if unhindered by projecting carbohydrate chains from Asn3 and Asn192, would also constitute potential anion binding regions. Relevant to these predictions is the three-dimensional structure of suramin itself. It is thought that sulfonate groups on the terminal naphthalene rings (Fig. 1) are the main sites for non-covalent interaction with proteins that, together with its overall symmetry, enables suramin to cross-link adjacent molecules and form intramolecular bridges. Although suramin is flexible and can rotate around several of the internal aromatic rings [20], in a non-hydrated low-energy state the calculated 'span' between terminal sulfonate groups is in the region of 25–30 Å. This approximates to the distance between several of the charged exosites on the surface of β-acrosin making it theoretically possible for several molecules of suramin to bind to one molecule of protein.

3.3. Stoichiometry of suramin binding and relationship to enzyme inhibition

Despite the similarity in sequence and structure of boar and ram sperm β-acrosins, there was a clear difference in the effect of suramin on their k_A values. Whereas boar β-acrosin was competitively inhibited by two molecules of suramin, ram β-acrosin showed an increase in specificity for the substrate caused by binding of a single suramin molecule. For this to occur, suramin must be binding to a region other than the active site and as a consequence induces a conformational change. This same mechanism may also be happening for boar β-acrosin, where the first suramin molecule increases the affinity for the second suramin molecule resulting in co-operative inhibition. It has been argued previously that suramin binding to proteins is unlikely to induce a conformational change [28]. However, the activation of ram β-acrosin clearly indicates that this is possible.

The suramin molecule that acts as a competitive inhibitor for boar β-acrosin presumably binds to a positively charged region near to the active site. Most of the charged residues are conserved between ram and boar β-acrosin, but Arg212 in boar β-acrosin is changed to a Glu in ram. It is possible, therefore, that this residue is important in binding one of the suramin molecules. Interestingly, human β-acrosin has a Val in this position which makes it difficult to predict whether suramin would act as a competitive inhibitor of the human protein or not, as this is a single charge difference from the boar β-acrosin, not a double charge difference as is the case for ram β-acrosin.

Despite the small increase in specificity constant of ram β-acrosin in the presence of suramin, the overall effect was inhibition. This inhibition was not complete, although it was consistently >90% (see b values in Table 1). The failure to achieve complete inhibition may be due to the substrate S2288 being a relatively small molecule. It is often the case that when

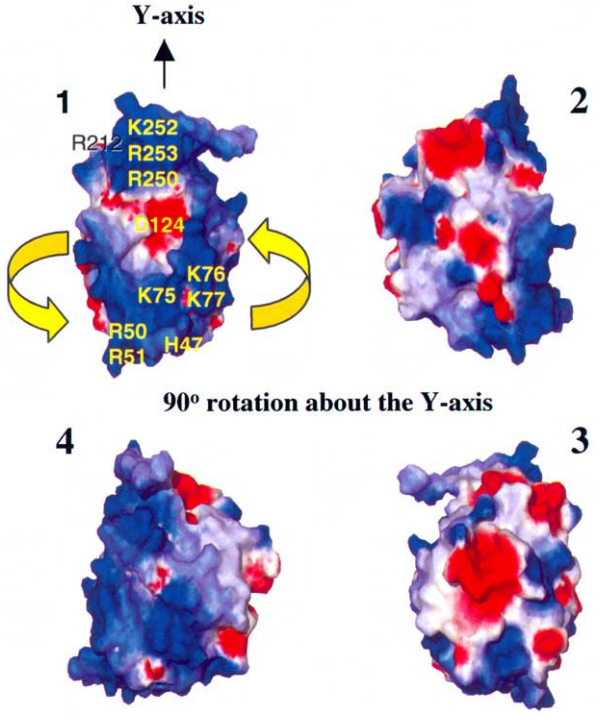

Fig. 3. Charge density model of boar β-acrosin illustrating the distribution of basic (coloured blue) and acidic (coloured red) residues on the surface of the protein. The relative positions of individual residues referred to in the text are shown numbered in yellow. The model is rotated counterclockwise in 90° steps about the *Y*-axis (1–4) to demonstrate the presence of potential docking sites for suramin.

only partial inhibition is observed with small substrates, complete inhibition occurs with large protein substrates [18].

In considering the number of suramin molecules bound to β-acrosin it is necessary to compare the K_{act} and K_{iu} values for ram β-acrosin (Table 1). The uncompetitive inhibition was fitted by an equation that describes cooperative inhibition by two molecules, which means that the second molecule binds to β-acrosin with higher affinity than the first molecule. Because the K_{iu} value obtained is significantly larger than the K_{act} value, and only one molecule is involved in increasing specificity, then the activating suramin molecule must be different from the two uncompetitive inhibitor suramin molecules, making a total of three suramin molecules binding to ram β-acrosin. By analogy, for boar β-acrosin the first competitive inhibitor molecule of suramin would differ from the two uncompetitive suramin molecules. The second competitive inhibitor suramin molecule only binds to boar β-acrosin and so must be different from the other three. Hence, four suramin molecules bind to boar β-acrosin and three to ram β-acrosin. It is not possible at present to predict precisely where the bound suramin molecules might lie, partly because of the uncertainties in modelling suramin due to its inherent flexibility and partly because of induced conformations brought about by binding of the first suramin molecule. Only by co-crystallising suramin with β-acrosin will it be possible to determine the precise binding sites.

In light of the above results, it may be speculated that suramin will interact with human β-acrosin in a similar manner and in so doing interfere with secondary sperm binding to the ZP [8]. Although this remains to be demonstrated, the possibility exists that safe mimetics of suramin have potential as antifertility agents.

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