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Kinetics of the BSA-dependent reaction of sorbic acid with mercaptoethanol and its inhibition by hex-3-enoic acid

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The ability of bovine serum albumin (BSA) to enhance the rate of the reaction between sorbic acid (SA) and mercaptoethanol (ME) is confirmed. The pseudo-first-order rate constant for the effect of SA concentration on rate ([ME] = 20 mM) is increased by a factor of about four when the reaction mixture contains 0·5% (w/v) BSA and [SA] < 1 mM. The enhancement of rate becomes smaller as SA concentration is increased, indicating 'saturation' kinetic behaviour. The formation of the reaction intermediate in BSA–SA–ME mixtures involves the binding of equimolar amounts of the reactants at up to some 90 sites on the BSA molecule. Hex-3-enoic acid (3HA) reduces the rate-enhancing effect of BSA; this is explained as a result of competition for the binding sites between 3HA and SA or the reaction intermediate.

INTRODUCTION

The rate of reaction between sorbic acid (SA) and a representative range of thiols (mercaptoethanol, mercaptoacetic acid, cysteine and glutathione) is enhanced by the addition of cationic and nonionic micelle-forming surfactants (Wedzicha & Zeb, 1990*a*) and bovine serum albumin (BSA) (Wedzicha & Zeb, 1991). In all these cases, the effect was interpreted as one of catalysis, involving the formation of a kinetically significant complex by hydrophobic and polar interactions between the surfactant micelle or BSA and the reactants. The effect of BSA was considered particularly significant as one of the few examples illustrating the need to include protein when modelling reactions between low molecular weight components of foods, even when the overall stoichiometry of the reaction does not include the protein molecule.

An alternative explanation for the enhancement of rate by BSA is the cleavage of the disulphide bonds of the protein by the low molecular weight thiol followed by a more rapid reaction of the released thiol groups with the SA. This possibility was considered by Wedzicha and Zeb (1991) by examining the stoichiometry (BSA : reactant) of the reaction intermediate. Thus, if the BSA concentration in a reaction mixture consisting of SA (10 mM) and mercaptoethanol (ME; 10 mM) is increased, the reaction becomes independent of BSA concentration when [BSA] > 2% (w/v). This corresponds to 0·31 mM BSA. (In the cited publication (Wedzicha & Zeb, 1991), the concentration of a 2% (w/v) solution of BSA was incorrectly stated as 31 μM. In consequence, the published concentration of disulphide bonds in relation to the concentration of ME was underestimated by an order of magnitude. The correct interpretation of the data is given here.) The concentration of ME equivalent to all 17 disulphide bonds of 0·31 mM BSA is 5·3 mM, which is the same order of magnitude as the concentration of ME in reaction mixtures.

Here we attempt to clarify the mechanism by measuring the stoichiometry (BSA : thiol and BSA : SA) of the reaction intermediate and the kinetics of the BSA-enhanced reaction in the presence of hex-3-enoic acid (3HA). This solute is expected to bind to BSA, as does any fatty acid, and perhaps will compete with SA for the binding sites. As the C=C bond in 3HA is not polarised (i.e. not conjugated to the carboxyl group), 3HA is unreactive towards thiols. The choice of 3HA represents a compromise for a structural analogue of SA. The single C=C bond imparts some rigidity to the molecule; on the other hand, hexanoic acid is very flexible. However, unlike SA, 3HA does not have the extensive delocalised system of electrons which renders the SA molecule flat. It was decided to use ME as the thiol rather than mercaptoacetic acid, cysteine or glutathione, to avoid the additional complications associated with the ionisation of acid groups.

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MATERIALS AND METHODS

Bovine serum albumin (fraction V, 96–99%) was obtained from Sigma Chemicals, Poole. For kinetic measurements, solutions of reactants and BSA in acetate buffer (0·2 M, pH 5·0, containing 0·1 mM EDTA) were mixed and made up to standard volumes with buffer to give reactant concentrations in the range 0–20 mM and [BSA] = 0, 0·5 and 1·0% (w/v). Reaction mixtures were dispensed into several 2-ml vials, with negligible headspace, and heated at 40 ± 0·1°C. Vials were withdrawn at timed intervals and analysed for thiol content by means of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman, 1959) as described by Wedzicha and Zeb (1991). Experiments to determine the effect of 3HA on rate involved preparation of the reaction mixtures described above but containing also 3HA (0–50 mM).

RESULTS AND DISCUSSION

The progress of the SA–ME reaction was followed by measuring the loss of thiol groups. Mercaptoethanol reacts with SA with 1:1 stoichiometry (Khandelwal & Wedzicha, 1990), with a rate-determining step which is of first order with respect to both SA and ME (Wedzicha & Zeb, 1990*b*). In the absence of BSA, the reaction is a nucleophilic addition of the thiolate ion to position 5 of the undissociated SA to form an anionic transition state. This subsequently reacts with H^+ to form the substituted 3HA (Khandelwal & Wedzicha, 1990).

The effects of SA concentration, at constant ME concentration, on the rates of loss of thiol groups in the absence of BSA and in the presence of 0·5 or 1% (w/v) BSA are shown in Fig. 1. In the absence of BSA, the expected first-order behaviour with respect to SA was observed. These data illustrate the magnitude of the rate-enhancing effect of BSA; at low SA concentration (e.g. <1 mM) the pseudo-first order rate constant (slope of the rate vs SA concentration curve) was increased by a factor of 3·9 when the reaction mixture contained 0·5% (w/v) BSA. The fact that this enhancement of rate increased slightly as the concentration of BSA was doubled indicates that the BSA–SA interaction responsible for the kinetic effect does not cause binding of all the SA. The overall second-order rate constant, k, in the rate equation for the BSA-independent reaction,

$$\text{rate} = k\,[\text{SA}]\,[\text{ME}]$$

is estimated to be 0·031 $M^{-1}\ h^{-1}$. The nonlinear rate vs concentration behaviour in the presence of BSA is indicative of 'saturation' kinetics, as might be observed in an enzyme-catalysed reaction. The rate of reaction became independent of SA concentration at [SA] >10 mM and >20mM for BSA concentrations of 0·5 and 1·0% (w/v) (75 and 150 μM), respectively. On the basis of this information, the best mechanism which may be proposed for the process of activation in the BSA–SA–ME reactions is an initial binding of SA to BSA followed by a reaction of the complex with ME, i.e.

$$\text{BSA} + \text{SA} \overset{\text{fast}}{\rightleftharpoons} \text{complex} \xrightarrow[\text{ME}]{\text{slow}} \text{product} + \text{BSA}$$

The saturation kinetic behaviour may be explained as the conversion of all the BSA to BSA–SA adduct when the concentration of SA is sufficiently high. Extrapolation of the tangent, drawn to the rate vs [SA] curve at the origin, to intersect with a horizontal line drawn at the value of the rate corresponding to the asymptote to the curve when SA concentration is large (i.e. 16 μM h^{-1}), gives the concentration of SA required to saturate the binding sites on the BSA molecule (Wedzicha & Zeb, 1990*a*). Using the data for 0·5% (w/v) BSA, this intersection was 6·7 mM, suggesting that there are 6·7/0·075 = 89 binding sites, at which the rate of the SA–ME reaction is enhanced. Unfortunately, the construction of the asymptote to the curve is subject to some uncertainty because the rate of reaction continued to increase with SA concentration as a result of the BSA-independent reaction involving free SA and ME. The measured stoichiometry nevertheless provides a good indication of the number of binding sites available.

Similar behaviour was observed when the concentration of ME was varied, keeping that of SA constant. To compare the shapes of the rate vs [ME] and rate vs [SA] graphs, in the presence of BSA, the rates obtained when the concentration of each reactant was varied, keeping that of the other reactant at 20 mM, were plotted against each other, as illustrated in Fig. 2. Each point on the graph corresponds to the two rates of reaction measured at the same concentration of both reactants the concentration of which was being varied; that obtained while varying the ME concentration is plotted along the y-axis and that obtained while varying SA concentration is plotted along the x-axis. The

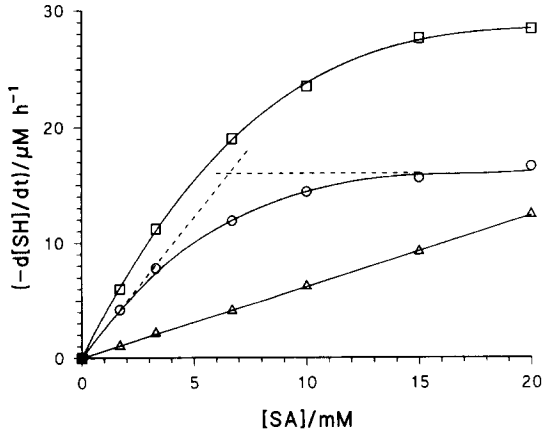

Fig. 1. The effect of sorbic acid (SA) concentration on the rate of loss of thiol groups in the SA–mercaptoethanol (ME) reaction in the absence of BSA (Δ) and presence of 0·5% (w/v) BSA (○) and 1% (w/v) BSA (□). Reaction conditions: [ME] = 20 mM, acetate buffer (0·2 M, pH 5·0), 40°C. The broken line shows the construction of tangent and asymptote to obtain the stoichiometry of the BSA–SA adduct.

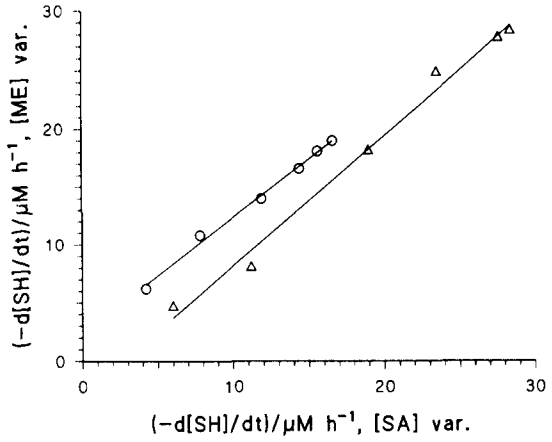

Fig. 2. Rate of reaction measured when the concentration of ME was varied with that of SA, kept constant at 20 mM, plotted versus the rate of reaction when [SA] was varied with [ME] = 20 mM. Successive points (with increasing rate) on each line were obtained with $[ME]_{var}$ = $[SA]_{var}$ = 1·7, 3·3, 6·7, 10, 15 and 20 mM for each run, in the presence of 0·5% (w/v) BSA (○) and 1% (w/v) BSA (Δ). Reaction conditions: acetate buffer (0·2 M, pH 5), 40°C.

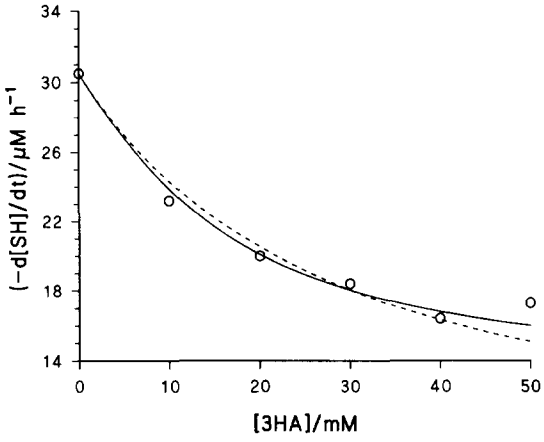

Fig. 3. Effect of hex-3-enoic acid (3HA) concentration on the rate of the SA–ME reaction in the presence of BSA (1% w/v). Experimental data are plotted as circles, with the lines calculated from the following rate laws: rate = $(k'[SA]_{bound} + k[SA]_{free})[ME]_{total}$ (continuous line) and rate = $k''[SA]_{bound}[ME]_{bound} + k[SA]_{free}[ME]_{free}$ (broken line), where k = 0·031 M^{-1} h^{-1}, k' = 0·120 M^{-1} h^{-1} and k'' = 0·262 M^{-1} h^{-1}. Reaction conditions: acetate buffer (0·2 M, pH 5), 40°C.

straight lines indicate that the kinetic behaviour of both reactants was similar, i.e. the shapes of the rate vs concentration curves were similar and both reactants appeared to saturate the binding sites at similar concentrations. This type of behaviour is indicative of a reaction where both reactants come together to form the reaction intermediate at the same sites on the BSA molecule.

The large number of molecules of each reactant binding per BSA molecule suggests that the behaviour cannot be attributed to the formation of thiol groups by cleavage of the 17 disulphide bonds of the protein. Sorbic acid could undergo hydrophobic interactions with BSA or bind ionically as the sorbate ion to positively charged residues on the protein. Spector *et al.* (1969) reported that fatty acid anions bind with high affinity (formation constant 10^5–10^6 M^{-1}) to three sites on the BSA molecule, and to a much larger number of sites (about 63) with an average formation constant of 10^3 M^{-1}. The concentration of SA required to achieve half of the maximum rate in the presence of 0·5% (w/v) BSA was about 3 mM; this is an estimate of the apparent dissociation constant of the BSA–SA adduct (Wedzicha & Zeb, 1990*a*); the apparent formation constant is, therefore, the reciprocal of this value, i.e. about 3×10^2 M^{-1}, and is reasonably consistent with the published estimate of 10^3 M^{-1} for the binding of saturated carboxylic acid molecules. However, it does not follow that all the carboxylic acid binding sites identified by Spector *et al.* (1969) represent those with rate-enhancing properties towards the SA–ME reaction.

Figure 3 shows that addition of 3HA to BSA–SA–ME reaction mixtures containing both reactants at concentrations (20 mM) close to those required to saturate the protein reduced the rate-enhancing effect of the BSA. At the maximum concentration of 3HA used (50 mM), the rate of reaction was about 16 μM h^{-1}, approaching that of 12·6 μM h^{-1} for the rate in the absence of BSA. It appears that 3HA competes with the reactants, or the reaction intermediate, for binding sites on the BSA molecule.

The possible competitive behaviour of 3HA towards SA can be modelled simply by considering the two simultaneous equilibria when both carboxylic acids react with the same site on the BSA molecule. We let the initial concentrations of binding sites, 3HA and SA be b, h and s, respectively, and the equilibrium concentrations of bound SA and 3HA be x and y, respectively. The dissociation constants, K_1 and K_2, of the SA–BSA and 3HA–BSA adducts, respectively, are given by

$$K_1 = (b - x - y)(s - x)/x$$
$$K_2 = (b - x - y)(h - y)/y$$

If r is the ratio K_2/K_1, the simultaneous equilibria may be described by

$$x^3(1 - r) + x^2(2rs - s + br - b + h + K_1r - K_1) + x(bs - 2bsr - hs - rs^2 - K_1rs) + bs^2r = 0$$

This cubic equation was solved for x after setting K_1 = 3 mM, b = 13·4 mM (i.e. 89 × 0·15 mM) and s = 20 mM, and the rate calculated from

$$\text{rate} = 0{\cdot}02\,k'x + k\,(0{\cdot}02 - x)\,0{\cdot}02$$

where k' is the second-order rate constant (0·12 M^{-1} h^{-1}) for the reaction of the BSA–SA adduct with ME. The second term accounts for the rate of the BSA-independent reaction involving free SA, including that which is displaced from the BSA binding sites by 3HA. In terms of reactant molecules, the rate equation is, explicitly,

$$\text{rate} = (k'[SA]_{bound} + k[SA]_{free})\,[ME]_{total}$$

It was assumed that [ME] = 0·02 M throughout the experiments and that the thiol was not bound to the BSA. The theoretical rate vs [3HA] curve, based on this model with r = 2·5 is shown in Fig. 3 to be a good fit to the data.

An alternative kinetic model is one where both SA and ME bind to BSA, and both are displaced by 3HA. If the reactants bind in equal amounts as suggested above, the rate of the reaction in the presence of 3HA can be calculated from

$$\text{rate} = k''x^2 + k(0{\cdot}02 - x)^2$$

where k'' is a second-order rate constant (0·262 M^{-1} h^{-1}). The rate equation with reactants shown explicitly is

$$\text{rate} = k''[\text{SA}]_{\text{bound}}\,[\text{ME}]_{\text{bound}} + k[\text{SA}]_{\text{free}}\,[\text{ME}]_{\text{free}}$$

The corresponding fit to the experimental data is also illustrated in Fig. 3, with $r = 0{\cdot}36$. Although the overall fit was not as good as for the first model, it was, nevertheless, very acceptable when considering that several assumptions are implicit in the approach used. In particular, all the binding sites on the BSA molecule are regarded as being equivalent. With this model the relative stabilities of the BSA–SA and BSA–3HA are reversed.

Notwithstanding the better fit of experimental data to the model in which a BSA–SA adduct undergoes a rate-determining reaction with ME (Fig. 3), the alternative model which involves the simultaneous binding of SA and ME to each site on the BSA molecule, followed by a rate-determining conversion of this intermediate to product, is more consistent with the overall kinetic evidence. Thus, it is proposed that the mechanism of the BSA-enhanced reaction of SA with ME is the interaction between BSA and the anionic intermediate formed as a result of addition of the thiolate anion to an undissociated SA molecule. The binding site on BSA reacts either with a preformed intermediate or there is a trimolecular reaction involving BSA, SA and ME. However formed, and whether or not its formation involves the making or breaking of covalent bonds, this trimolecular reaction intermediate undergoes a rate-determining change causing the thiol to become unavailable when analysed by means of DTNB at pH 7·0. The function of BSA in enhancing the rate of the SA–ME reaction could be analogous to the effects of nonionic and cationic surfactants on this reaction (Wedzicha & Zeb, 1990*a*), which are still unexplained. However, the numerous positively charged groups on the BSA molecule could stabilise the anionic reaction intermediate or perhaps assist in protonation of the intermediate to give the expected reaction product.

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