The Adsorption of Lactoperoxidase to Glass

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Lactoperoxidase (LP, E.C.1.11.1.7) catalyzes the oxidation of thiocyanate by hydrogen peroxide to the antibacterial hypothiocyanite.[1-3] LP and thiocyanate are excreted in the saliva, and peroxide is generated by bacteria normally occurring in the mouth.[4] LP is irreversibly adsorbed to tooth enamel,[5] which may locally enhance the antibacterial effect. The mode of binding is incompletely known. To penetrate further the binding mechanisms, we have studied the adsorption of LP to a simpler, non-biological surface, glass.

Materials and methods. LP[6] (from milk) and horseradish peroxidase C[7] were isolated to give $A_{Soret}/A_{280} = 0.93$ and 3.40, respectively. Horse heart cytochrome *C* was purchased from Sigma. LP was assayed kinetically with 2 mM dicarboxidine [$\gamma\gamma'$-(diamino-3,3'-bifenylylenedioxy)dibutyric acid] as chromogen and 50 μM H_2O_2 as oxidant.[8,9] The activity is expressed as $\Delta A_{440} \times s^{-1}$. Horseradish peroxidase was determined spectrophotometrically from $\varepsilon_{mM} = 102\ cm^{-1}$ at 402 nm.[10] Pyrex® ballotini (diameter 0.2 mm, density 2.4 $g \times cm^{-3}$) were pretreated by submersion in 1 M NaOH or 1 M HCl for one hour at room temperature, washed with distilled water until the washing showed pH ~ 7, and dried overnight at 105 °C. During this treatment the solute cations will to some extent exchange with the surface of the glass but not penetrate very deeply. There will be only little difference in etching after the two treatments because of the short duration. Ballotini are manufactured by a melting procedure that gives a smooth surface, but some beads are broken or irregular in shape.

Beads were gently shaken for one hour with a peroxidase solution, and then the "residual" (not adsorbed) peroxidase was immediately assayed. "Bound LP" was obtained as the difference between residual activities and activities in controls with peroxidase in identical vials but without beads. The shaking itself did not inactivate LP.

Unless otherwise stated 50 mM sodium phosphate, pH 6.9, and 25 °C were used for all adsorptions, assays *etc.* Given values are based on triplicate determinations on 5–35 μl of the LP solutions.

Results. Alkali-treated glass adsorbed about twice as much LP as acid-treated glass did. Only alkali-treated beads were used in the sequel.

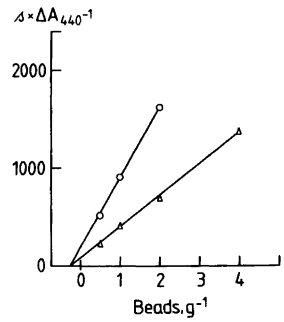

Fig. 1. A double inverse plot of peroxidase activity representing bound peroxidase (*cf.* Methods), and weight of beads when 1.5 ml of 1 μM LP in buffer were exposed to an increasing number of beads. The activity was determined on 10 (○) and 20 (△) μl samples.

Desorption by various media was tested on 1 g of beads, preequilibrated with 2.0 ml of 1 μM LP in buffer and subsequently washed with distilled water. Elution by sodium phosphate, pH 6.9, depended upon the ionic strength, 50 mM removing no LP and 300 mM about 25 % of the bound LP. Reinforcement of the weak buffer with 3.5 M ethanol had no effect whereas the presence of 1 M urea in this buffer eluted about 5 % of the LP. The enzyme is stable in both solutions.

When increasing amounts of beads were added to a constant amount of LP the residual activity decreased in such a manner that a plot of (bound LP)$^{-1}$ against (weight of beads)$^{-1}$ formed a straight line (Fig. 1).

The amount of LP bound per unit area of glass is obtained from Fig. 2. The extrapolation of the rectilinear slope to zero activity shows that 0.5 g of

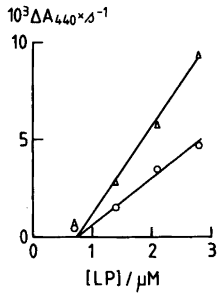

Fig. 2. Peroxidase activity remaining in solution when 0.5 g of beads is equilibrated with 1.5 ml of LP of various concentrations in buffer. The activity was determined on 8 (△) and 5 (○) μl samples.

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beads becomes saturated with LP when exposed to 1.5 ml of 0.7 μM LP, which gives a binding capacity of 2.1 nmol of LP per gramme of beads.

One gramme of beads adsorbed no horseradish peroxidase from 2 ml of a 1 μM solution in buffer, whereas under the same conditions Fe(III)cytochrome C was completely removed by the beads.

Discussion. Glass contains silicon-bound bridging and non-bridging oxygen atoms. The former can accept hydrogen bonds, and the latter carry a negative charge and act as a cation exchanger. Hydrolysis increases the ratio $Si-O^-/Si-O-Si$ and is favoured by alkali. LP is a basic protein containing 71 lysine+arginine residues.[11] Free electrophoresis showed pI = 6.9 in 0.1 M phosphate buffer and 8.0 in 0.1 M veronal; pI in phosphate varied with the ionic strength.[12] Prequisites for ionic bonds between LP and glass are thus at hand.

The effect of the ionic strength on the desorption of LP from glass, and the difference between alkali- and acid-treated beads, points at ionic bonds between LP and glass. There may also be a small contribution from hydrogen bonds.

The rectilinear relationship in Fig. 1 is compatible with a mechanism where all LP molecules are equally firmly and reversibly bound. The bonding capacity of 2.1 nmol of LP per gramme of beads corresponds to 1.3×10^{10} molecules per bead. The mean glass area available to one LP molecule, or to its projection on the glass, then becomes 970 Å^2, assuming a monolayer of LP molecules; the assumption is justified by Fig. 1. A spherical molecule of 78 400 dal with a partial specific volume of 0.725 ml $\times$ g^{-1} [11] and carrying a hydration mantle of 20 % (w/w) would require an area of 3100 Å^2 at closest packing. Sedimentation and diffusion analyses on LP gave the axial ratio of 5.6 for a prolate ellipsoid.[11] Such a conformation of this molecular weight and hydration requires a minimum area of 980 Å^2. This is in good agreement with the observed value of 970 Å^2 even with some allowance for the irregularities of the beads. The present result confirms the elongated form of the LP molecule and indicates the existence of positive charge(s) at one of its ends.

LP is very firmly bound to octyl-Sepharose® by what must be hydrophobic bonds.[6] The molecule is thus equipped for binding to negatively charged as well as to hydrophobic areas. Interestingly, its reactions with octyl-Sepharose®, Amberlite XE-64-N^+H_4 and glass equal those of a peripheral membrane protein, cytochrome *C*. Horseradish peroxidase is inert to the three adsorbants.

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