Cetyltrimethylammonium Ion Stabilization of Tribromide Ion from Peroxidase Reaction

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Cetyltrimethylammonium bromide (CTABr) is commonly used for the solubilization of membrane-bound peroxidases. In the lactoperoxidase/H₂O₂-catalyzed oxidation of Br⁻, only weak light absorption is seen. It becomes much more intense when CTA⁺, at concentrations far below those used for solubilization, is present. Lactoperoxidase oxidizes Br⁻ to OBr⁻/HOBr, which post-enzymatically yields Br₂ and Br₃⁺, the latter strongly UV-absorbing. CTA⁺ stabilizes the concentration of the tribromide ion. The effect is seen only above a critical concentration of CTA⁺, which suggests that ordered detergent structures are involved. In spectrophotometric studies of reactions with CTABr-solubilized peroxidases there is a risk of interference from Br₃⁺ in the ultraviolet region.

The cationic detergent N,N,N-trimethyl-1-hexadecaninium bromide (cetyltrimethylammonium bromide, CTABr) is commonly used to solubilize membrane-bound peroxidases, typically at a concentration of 0.5 % w/v.¹ When exposed to lactoperoxidase (LP) and H₂O₂, CTABr gives rise to an intense but transitory light absorption in the ultraviolet range,² even at concentrations a few percent of those used for the solubilization. The absorption is 50–80 times stronger than that produced by LP–H₂O₂–Br⁻ without detergent. Thus, remaining traces of CTABr may introduce an error in spectrophotometric studies with these peroxidases, and it was therefore of interest to analyze the intense UV light absorption.

Materials and methods

Bovine LP (EC 1.11.1.7)³ and horse-radish peroxidase C2 (EC 1.11.1.7)⁴ were isolated as described. CTABr (Merck, Darmstadt) was recrystallized three times from warm water. Cetyltrimethylammonium chloride (CTACl, Fluka) and Triton® X-100 (BDH) were used as purchased. Sodium hypobromite was prepared in solution from bromine (A. G. Riedel de Haën) vapour and ice-cold 0.1 M NaOH, and ethyl hypobromite (CH₃CH₂OBr) from Br₂ and ethanol in CCl₄. Ten transfers of bromine vapour with a constricted pipette gave an average quantity of 16.2±0.7 µmol (range 14.7–17.4 µmol) per transfer, as assayed optically using the value ε₄₁₃ = 212 M⁻¹ cm⁻¹ for Br₂ in CHCl₃.⁵ Cuvette temperatures in the Beckman DU-7 spectrophotometer were measured with a Comark type 1601 electronic Cr-Al thermometer. The experiments with Triton® X-100 were performed with a 2 mm cuvette.

Results

When H₂O₂ was added to LP in 2 mM CTABr, an intense light absorption with maximum at 271 nm appeared instantaneously. It lasted for only a few seconds at room temperature and neutral pH, but could conveniently be studied at low temperature and low pH. Its initial rate of appearance, but not disappearance, was proportional to [LP]. The absorption maximum decayed in a non-ordered manner with half-times of ≈120 s at 1°C and ≈20 s at 9°C (Fig. 1). An unbuffered reaction mixture increased in pH during the reaction. Lactoperoxidase oxidizes iodide and bromide. Horse-radish peroxidase, which can oxidize iodide but not bromide, generated no light-absorbing material from H₂O₂ and CTABr.
LP–H₂O₂–KBr produced only a weak absorption at 250–290 nm, and LP–H₂O₂–CTACl none. The presence of CTACl at micellar concentration during the reaction with KBr exactly reproduced the reaction with CTABr. The addition of CTACl (>0.1 mM) to “inorganic” HOBr in water shifted $A_{\text{max}}$ from 261 nm to 271 nm, with a 10-fold increase in intensity which further increased at higher [CTABr] (Fig. 2). The pH increased simultaneously; CTACl itself did not alter pH. Consequently, Br⁻ is the substrate and CTañ a prerequisite for the appearance of the intense absorption. The CTABr concentration required for the intense absorption was critical, its minimum being 0.39 mM at 0.5 °C, 0.46 mM at 4 °C, and 0.8 mM at 30 °C. The critical CTABr concentration thus varied with temperature in the same way as the critical micelle concentration. The possibility of CTañ micelle formation was explored by spectrophotometry of the anionic dye erythrosin. At [CTañ] ≥0.1 mM the position of the absorption maximum shifted from 548 to 541 nm. Thus, the shift occurred at a concentration lower than the critical concentration of ≈0.5 mM found above, but coincides with the break-point in the model system with HOBr–CTACl (cf. Fig. 2). An initial shift, from 527 to 548 nm, occurred at [CTañ] ≤5 μM; this concentration is

Fig. 1. Absorbance vs. time for the LP–H₂O₂–CTABr reaction, at 1 °C (—) and at 9 °C (—–), and for the reaction with 2 mM KBr (—–, 1 °C) instead of CTABr. Experimental conditions: 50 mM sodium citrate (pH 4.0), 40 nM LP, 2.0 mM CTABr, 100 μM H₂O₂. The reactions were monitored at the wavelengths of maximum absorption. The insert represents the KBr reaction with expanded absorbance scale. H₂O₂ was added and mixed at 2 to 6 s.

Fig. 2. Spectral changes occurring upon mixing CTACl and HOBr. 200 μl of 4.0 mM unbuffered (pH 12.6) hypobromite with an equal concentration of bromide in 0.1 M NaOH were added to a cuvette containing 0–2.6 mM CTACl in 2 ml of 50 mM sodium phosphate (pH 7.0, 25 °C). The absorbance increased rapidly, reaching ~97 % of its maximum within 2 min; it then levelled off and eventually decreased slowly. The 2-min absorbance is given at 271 nm (○) and at wavelength maximum (▼). The wavelength of the absorption maximum is given in the bottom curve (○).
equal to the erythrosin concentration and the shift may represent some stoichiometric interaction. Mukerjee and Mysels have criticized the use of dyes for the determination of critical micelle concentration.9,10

Bromide ions are substrates for LP and some other peroxidases,11 but not for horse-radish peroxidases,12,13 with hypohalite as the final product.14,15 Hypobromite and Br₂ generate the tribromide ion, Br₃⁻; this species absorbs strongly in the UV region (Table 1). The enhancement of the absorption by CTA⁺ might be the result of either a solvent effect on HOBr (pKₐ 8.7) or a stabilization of HOBr or Br₂⁻. The position of λ_{max} for the HOBr analogue ethyl hypobromite was found to depend upon the solvent: 266 nm in ethanol, 272 nm in chloroform, 273 nm in cyclohexane and 275 nm in carbon tetrachloride. Accordingly, a bathochromic shift would be expected if HOBr had been transferred from water to the less polar environment of the detergent hexadecyl moiety. However, CHCl₃ failed to extract any light-absorbing material from the reaction mixture at pH 7 or pH 2, contrary to statements to this effect.16 Whereas LP-H₂O₂-CTABr gave A₂₇₁ = 1.5, there was hardly any spectral change with LP-H₂O₂-KBr and 4 mM Triton® X-100. Finally, the absorptivity of HOBr is not very high (Table 1). Hence, a transfer of HOBr to a lipophilic milieu cannot be the cause of the intense light absorption and the bathochromic shift.

Upon mixing 4 mM Br₂ and 2 mM KBr at pH 4 and 20°C in the absence of detergent, the absorbance reached 0.5 within mixing time, with maximum at 271 nm. It then decayed and shifted towards 264 nm. Under identical conditions Br₂ and CTABr gave A₂₇₁ > 10 (estimated geometrically). This absorption also decayed but the maximum remained at 271 nm. Hence, Br₂ and Br⁻ form Br₃⁻ whether CTA⁺ is present or not, but the detergent stabilizes the absorption.

**Discussion**

The formation of Br₃⁻17 is possible under the conditions of the experiment:

\[
\text{Br}^- + \text{H}_2\text{O}_2 \rightarrow \text{OBr}^- + \text{H}_2\text{O} \quad (1)
\]

\[
\text{H}^+ + \text{OBr}^- \rightleftharpoons \text{HOBr} \quad (2)
\]

\[
\text{HOBr} + \text{H}^+ + \text{Br}^- \rightleftharpoons \text{Br}_2 + \text{H}_2\text{O} \quad (3)
\]

\[
\text{Br}_2 + \text{Br}^- \rightleftharpoons \text{Br}_3^- \quad (4)
\]

Reactions (1) and (2) are fast and proceed far to the right under the actual conditions. At low pH, reaction (3) favours Br₂ formation; the reverse reaction between Br₂ and H₂O is slow. Reaction (4) has [Br₂][Br⁻]/[Br₃⁻] = 0.062 M, with equilibrium attained in ≈100 ns and the forward reaction rate close to the diffusion limit.18 As long as (1) and (2) generate HOBr the concentration of Br₃⁻ remains elevated, but when (1) ceases, disproportionation of HOBr (OBr⁻ + 2HOBr), and possibly other reactions, deprive the system of this species and reaction (4) is reversed. In all probability reaction (1) is faster at pH 4 than at pH 7,19 whereas the disproportionation is faster close to pH = pKₐ(8.7). As a consequence, the formation of Br₃⁻ becomes much more pronounced at pH 4 than at pH 7. The observed increase in pH is in accord with this reaction pattern.

The absorptivity of Br₃⁻ in aqueous solution has been determined as 39 mM⁻¹ cm⁻¹ at 270 nm and as 36.4 mM⁻¹ cm⁻¹ at 278 nm.16 Using the higher value also when CTA⁺ is present, the peak proportion of Br₃⁻ was 40% (Fig. 1, 1°C), calculated on the basis of available H₂O₂. With 9 mM KBr, 3.1 mM CTACl, 80 mM LP and

**Table 1. Spectral characteristics of some bromine-containing compounds.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Absorptivity/ mM⁻¹ cm⁻¹</th>
<th>λ/nm</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOBr</td>
<td>0.1</td>
<td>260 max</td>
<td>24</td>
</tr>
<tr>
<td>OBr⁻</td>
<td>0.2</td>
<td>333 max</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>271 slope</td>
<td>24</td>
</tr>
<tr>
<td>Br⁻</td>
<td>0</td>
<td>260-270</td>
<td></td>
</tr>
<tr>
<td>Br₂</td>
<td>0.164</td>
<td>392 max</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>&lt;0.1</td>
<td>271 slope</td>
<td>25</td>
</tr>
<tr>
<td>Br₂⁻ᵇ</td>
<td>9.6±0.8</td>
<td>360 max</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>1.3±0.3</td>
<td>270 slope</td>
<td>20</td>
</tr>
<tr>
<td>Br₃⁻ᶜ</td>
<td>39±2.0</td>
<td>270 max</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>36.4±1.6</td>
<td>278</td>
<td>16</td>
</tr>
<tr>
<td>CH₃CH₂OBr⁺</td>
<td>0.08</td>
<td>276 max</td>
<td>24</td>
</tr>
<tr>
<td>BrO₃⁻</td>
<td>0.001</td>
<td>270 slope</td>
<td>26</td>
</tr>
</tbody>
</table>

*All in water except for CH₃CH₂OBr, which was dissolved in CCl₄.*  
*Half-life <100 µs.*  
*Stable for at least 2 min in sulfuric acid, pH 2.*  

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75 μM H₂O₂, the peak proportion became 98% at pH 4 and 1°C. The higher ratio in this case is to be expected from reactions (1)–(4).

Micelles and pre-micellar aggregates can accelerate bimolecular reactions by means of concentration mechanisms.²¹ Br₂ is more soluble and more stable in alkanes than in water. An increase in CTA⁺ micelle concentration enlarges the phase border area, which facilitates Br₂ transfer to the hydrocarbon phase. The charged group in CTA⁺ may concentrate Br⁻ ions; CTA⁺ shows a preference for Br⁻ over Cl⁻,²¹ and in the vicinity of a micellar or submicellar structure a low bulk phase pH will augment [Br⁻] at the expense of [OH⁻]. The locally increased [Br⁻] accelerates reactions (3) and (4). Reaction (1) is less likely to be affected, since the volumes of the micelle (30–500 nm³)²² and the LP molecule (180 nm³)²³ are of the same order of magnitude. Besides facilitating reactions (3) and (4), CTABr may exert its effect by preserving Br₅⁻.⁸

Peroxidases catalyze transformations of a variety of substances, some of which absorb light in the ultraviolet region. If trace amounts of CTABr remain after solubilization of a peroxidase, optical studies of e.g. phenol and enediol reactions may be seriously perturbed by the enhanced absorption of Br₂ in the UV-region. This is particularly the case in studies of the more acidic halogeno- and nitrophenols, since the protonated form and not the base is the actual substrate.

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