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/H_2O_2-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 Br_2 and Br_3^-, 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 Br_3^- in the ultraviolet region.

The cationic detergent *N,N,N*-trimethyl-1-hexadecanaminium bromide (cetyltrimethylammonium bromide, CTABr) is commonly used to solubilize membrane-bound peroxidases, typically at a concentration of 0.5 % *w/v*.[1-4] When exposed to lactoperoxidase (LP) and H_2O_2, CTABr gives rise to an intense but transitory light absorption in the ultraviolet range,[5] even at concentrations a few per cent of those used for the solubilization. The absorption is 50–80 times stronger than that produced by LP–H_2O_2–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)[6] and horse-radish peroxidase C2 (EC 1.11.1.7)[7] 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 (CH_3CH_2OBr) from Br_2 and ethanol in CCl_4. Ten transfers of bromine vapour with a constriction 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 $\varepsilon_{413} = 212$ M^{-1} cm^{-1} for Br_2 in $CHCl_3$.[8] 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 H_2O_2 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 H_2O_2 and CTABr.

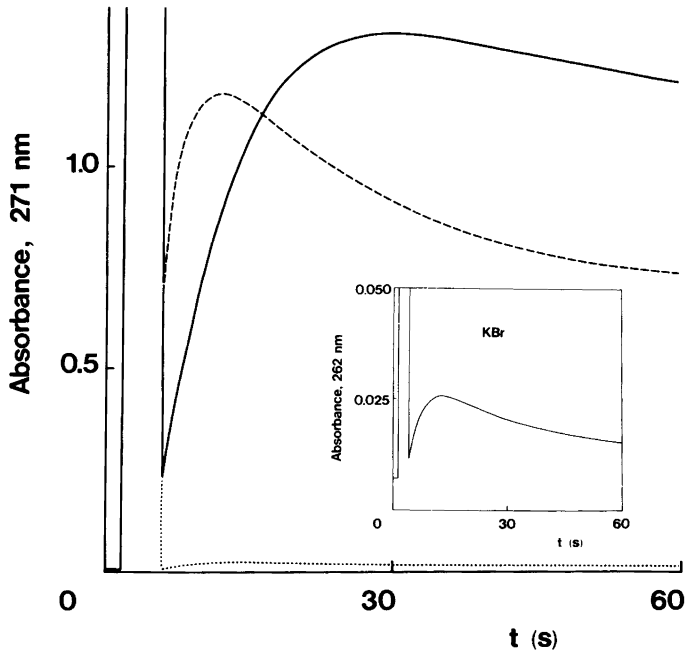

Fig. 1. Absorbance vs. time for the LP–H_2O_2–CTABr reaction, at 1 °C (——) and at 9 °C (– –), and for the reaction with 2 mM KBr (---, 1 °C) instead of CTABr. Experimental conditions: 50 mM sodium citrate (pH 4.0), 40 nM LP, 2.0 mM CTABr, 100 μM H_2O_2. The reactions were monitored at the wavelengths of maximum absorption. The insert represents the KBr reaction with expanded absorbance scale. H_2O_2 was added and mixed at 2 to 6 s.

LP–H_2O_2–KBr produced only a weak absorption at 250–290 nm, and LP–H_2O_2–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_{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 CTA^+ 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 CTA^+ micelle formation was explored by spectrophotometry of the anionic dye erythrosin. At $[CTA^+] \geq 0.1$ 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 $[CTA^+] \leq 5$ μM; this concentration is

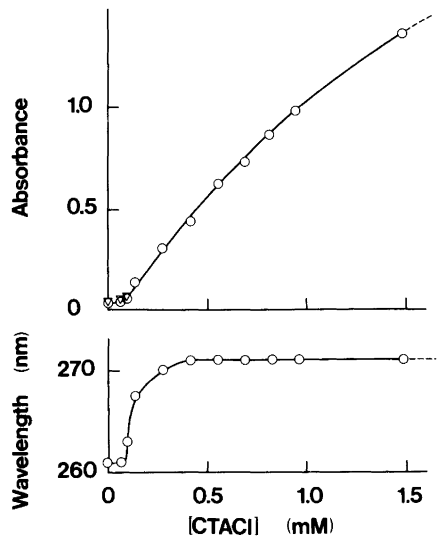

Fig. 2. Spectral changes occurring upon mixing CTACl and HOBr. 200 μl of 4.0 mM unbuffered (pH 12.6) hypobromite with an equal concentration of bromide in 0.1 M NaOH were added to a cuvette containing 0–2.6 mM CTACl in 2 ml of 50 mM sodium phosphate (pH 7.0, 25 °C). The absorbance increased rapidly, 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 Br_2 generate the tribromide ion, Br_3^-; 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 (pK_a 8.7) or a stabilization of HOBr or Br_3^-. The position of A_{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, $CHCl_3$ 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–H_2O_2–CTABr gave $A_{271} = 1.5$, there was hardly any spectral change with LP–H_2O_2–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.

Table 1. Spectral characteristics of some bromine-containing compounds.[a]

Species	Absorptivity/ mM^{-1} cm^{-1}	λ/nm	Ref.
HOBr	0.1	260 max	24
OBr^-	0.2	333 max	24
	0.03	271 slope	24
Br^-	0	260–270	
Br_2	0.164	392 max	25
	<0.1	271 slope	
$Br_2^{\cdot-}$ [b]	9.6±0.8	360 max	20
	1.3±0.3	270 slope	20
Br_3^- [c]	39 ±2.0	270 max	20
	36.4±1.6	278	16
CH_3CH_2OBr[a]	0.08	276 max	24
BrO_3^-	0.001	270 slope	26

[a]All in water except for CH_3CH_2OBr, which was dissolved in CCl_4. [b]Half-life <100 μs.[18] [c]Stable for at least 2 min in sulfuric acid, pH 2.[27]

Upon mixing 4 mM Br_2 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 conditons Br_2 and CTABr gave $A_{271} > 10$ (estimated geometrically). This absorption also decayed but the maximum remained at 271 nm. Hence, Br_2 and Br^- form Br_3^- whether CTA^+ is present or not, but the detergent stabilizes the absorption.

Discussion

The formation of Br_3^- [17] is possible under the conditions of the experiment:

$$Br^- + H_2O_2 \xrightarrow{LP} OBr^- + H_2O \quad (1)$$

$$H^+ + OBr^- \rightleftharpoons HOBr \quad (2)$$

$$HOBr + H^+ + Br^- \rightleftharpoons Br_2 + H_2O \quad (3)$$

$$Br_2 + Br^- \rightleftharpoons Br_3^- \quad (4)$$

Reactions (1) and (2) are fast and proceed far to the right under the actual conditions. At low pH, reaction (3) favours Br_2 formation; the reverse reaction between Br_2 and H_2O is slow. Reaction (4) has $[Br_2][Br^-]/[Br_3^-] = 0.062$ 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 Br_3^- 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 $pH = pK_a(8.7)$. As a consequence, the formation of Br_3^- 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 Br_3^- in aqueous solution has been determined as 39 mM^{-1} cm^{-1} at 270 nm[20] 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 Br_3^- was 40 % (Fig. 1, 1 °C), calculated on the basis of available H_2O_2. With 9 mM KBr, 3.1 mM CTACl, 80 nM LP and

75 μM H_2O_2, 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] Br_2 is more soluble and more stable in alkanes than in water. An increase in CTA^+ micelle concentration enlarges the phase border area, which facilitates Br_2 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 nm^3)[22] and the LP molecule (180 nm^3)[23] are of the same order of magnitude. Besides facilitating reactions (3) and (4), CTABr may exert its effect by preserving Br_3^-.[18]

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 Br_3^- in the UV-region. This is particularly the case in studies of the more acidic halo- and nitrophenols, since the protonated form and not the base is the actual substrate.

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