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Biliverdin as an electron transfer catalyst for superoxide ion in aqueous medium

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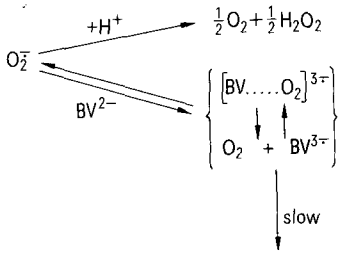
Summary. Stopped flow experiments gave evidence of the formation of a biliverdin-superoxide complex and/or a biliverdin radical anion by reaction of aqueous O_2^- with biliverdin. Such transient species are likely intermediates both in the bleaching of biliverdin, during exposure to the aerobic xanthine oxidase reaction, and in the reduction of ferricytochrome *c* under the same conditions.
Key words. Biliverdin; superoxide ion; cytochrome *c*; stopped flow technique; xanthine oxidase; superoxide dismutase.

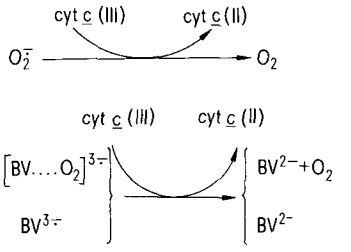
Although the bleaching of biliverdin (BV)[1] during exposure to the aerobic xanthine oxidase reaction has been reported by Fridovich[2], the chemical processes involved remain to be clarified. Recently we found that BV (and its dimethyl ester) interacts rapidly with KO_2 in DMSO, giving rise to a reversible 1:1 adduct[3]. This prompted us to investigate whether a similar charge-transfer complex could be the actual intermediate in the reaction of BV with enzymatically generated superoxide[4]. In this paper we give spectroscopic evidence of the formation of radical anions, such as the complex $[BV\cdots O_2]^{3-}$ or BV^{3-} or both, as likely transient intermediates in the bleaching of BV by aqueous O_2^- and in the BV-catalyzed reduction of ferricytochrome *c* by the same reagent. In studying the system BV/KO_2 in DMSO a thermodynamic approach (i.e. chemical equilibrium determination) was followed[3]. Such a method, however, appeared to be unsuitable in the case of aqueous media since the superoxide ion undergoes a rapid dismutation (to O_2 and H_2O_2) in protic solvents[5]. For this reason a kinetic approach, based on a stopped flow technique, was chosen. When BV[6], dissolved in an oxygenated solution of xanthine, was mixed with an oxygenated solution of xanthine oxidase in stopped flow apparatus, a transient species A could be detected showing an electronic absorption maximum at 730 nm (figs 1 and 2).

Equal volumes of two solutions containing xanthine-oxidase in phosphate buffer pH 7.6 and a mixture of xanthine and biliverdin in DMSO were mixed in a stopped flow apparatus (Nortech Laboratories Limited, England, model FPX-1; mixing time 5 ms) according to the conditions described in the captions of the figures. The transient signals were recorded by means of a Tektronix model 5115 oscilloscope and a Polaroid camera. The base line of the starting spectrum was practically restored after the decay of A (within 200 ms), indicating that the BV bleaching occurs on a completely different time scale (cf. Robertson Jr and Fridovich[2]). Formation of A was not observed when deoxygenated solutions were used, and the concentration of A was lowered to zero by the addition of increasing amounts of bovine erythrocyte superoxide dismutase. Thus, figure 2 can be regarded as a pre-steady state of the reaction of BV with O_2^- generated by the system xanthine/xanthine oxidase/oxygen.

It must be pointed out that the reported spectrum of the BV radical anion at basic pH (BV^{3-})[7] fits the spectrum of A nearly exactly[8]. However, one cannot rule out that A is a charge-trans-



Scheme 1. bleaching of BV



Scheme 2.

fer complex, viz. $[BV\cdots O_2]^{3-}$, like that formed by interaction of BV with KO_2 in DMSO (which shows a comparable spectrum)[3]. We cannot even rule out that the charge-transfer complex is actually formed to give successively dioxygen and BV^{3-} through a rapid internal electron transfer (see scheme 1). Whatever the structure of A may be, it is noteworthy that its formation seems to be associated with a rate enhancement of reactions involving the superoxide ion as a one-electron reductant, as shown by the following findings.

The reduction of ferricytochrome *c* by superoxide has been largely utilized as an indirect method to detect and to measure production and decay of O_2^- in aqueous solution[2,4]. The addition of BV to a system containing cytochrome *c*, xanthine, xanthine oxidase and molecular oxygen resulted in a sharp increase of the rate of cytochrome *c* reduction, as shown in figure 3[9]. Curves a and b in figure 3 differ when BV = 0 because the reduction of cytochrome *c* by O_2^- is first order in cytochrome *c* under the experimental conditions. The rate constant for the reaction of superoxide ion with cytochrome *c* has been estimated as 1.1×10^5–1.1×10^6 M^{-1} s^{-1}, whereas larger rate constants have been reported in the oxidation of free radicals by ferricytochrome *c*[10].

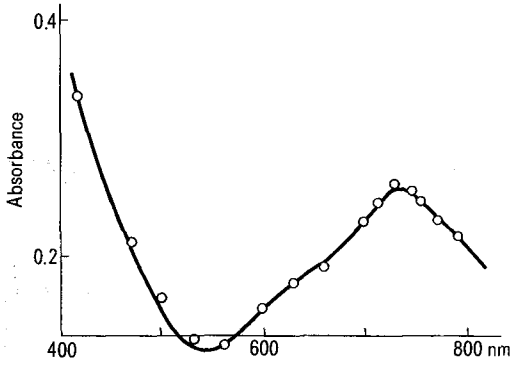


Figure 1. Difference spectrum of A. Absorbances at different wavelenghts were recorded 45 ms after mixing a solution of xanthine and BV with a solution of xanthine oxidase (i.e. at the maximum concentration of A; see fig. 2). Conditions: T = 21 °C, phosphate buffer 0.03 M, pH 7.6, O_2 at saturation; final concentrations: xanthine 10^{-3} M, BV 10^{-4} M, xanthine oxidase 10 mU/ml.

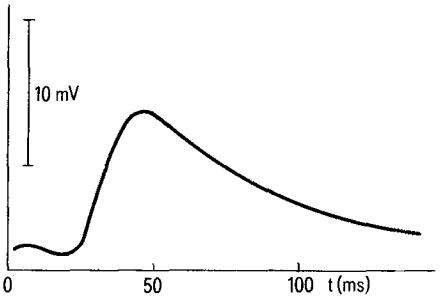


Figure 2. Oscillogram recorded at 730 nm from a stopped flow experiment. Conditions as in figure 1.

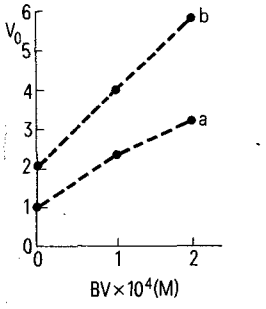


Figure 3. Initial rate of cytochrome *c* reduction (in arbitrary units) vs BV concentration. Conditions: T = 21 °C, phosphate buffer 0.1 M pH 7.6, xanthine 10^{-3} M, xanthine oxidase 4 mU/ml, O_2 at saturation, cytochrome *c*: (a) 2×10^{-5} M, (b) 4×10^{-5} M.

The different time scale for the reduction of ferricytochrome *c* by superoxide, which is accomplished within a few minutes, and the bleaching of biliverdin (lasting more than 1 h, $k = 7 \times 10^3$ M^{-1} s^{-1}) rules out that any radical fragment deriving from the latter process can be responsible for the increase of the rate of the former. Thus, a plausible explanation of this fact is depicted in scheme 2, pivoting on the transient species A and taking into account all the hypotheses mentioned above about the nature and the origin of A. The following assumptions can be made: a) a steady state concentration of A is reached after ca. 200 ms after starting the reaction producing superoxide; b) superoxide reacts with BV more rapidly than with ferricytochrome *c*; c) ferricytochrome *c* is reduced more rapidly by A than by superoxide.

A steady state concentration of A could also explain the slow bleaching of BV (scheme 1): if A is the BV radical anion, this is expected to disproportionate to give BV and bilirubin, which is rapidly destroyed by superoxide[2]. If A is a charge-transfer complex, it could be bleached via hydrogen abstraction as shown previously[3]. Thus figure 2 can be interpreted as follows. The upward side of the curve corresponds to the direct reaction of BV^{2-} with O_2^-, whereas the downward side is related to at least two processes: the reverse reaction of the equilibrium between BV^{2-} and O_2^-, whose driving force is given by superoxide dismutation, and the bleaching of A. After a brief pre-steady state, the steady state concentration of A is obtained, but it is so low that it cannot be estimated by its absorption. This interpretation is reinforced by the observation that, introducing another consecutive reaction of A by adding ferricytochrome *c*, the maximum concentration of A (observable as in fig. 2) is lowered.

While it is well known that the reactivity of superoxide ion can be affected by its complexation with metal ions[5], its interaction with a biological molecule (biliverdin) represents, to our knowledge, the first example of a reactivity enhancement by the intermediacy of a rapidly formed radical species.

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