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Preparation of reduced bovine Cu,Zn superoxide dismutase

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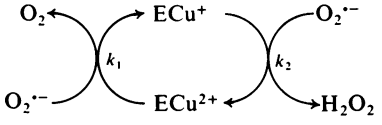
N.m.r. and e.p.r. were used to measure the oxidation state of copper in Cu,Zn superoxide dismutase treated with reducing agents such as $NaBH_4$, $K_4Fe(CN)_6$, $Na_2S_2O_4$ and H_2O_2. The activity and the electrophoretic pattern of the treated enzyme were also studied. On the basis of the reducing ability and of the absence of inactivating effects, $NaBH_4$ was the most suitable reducer of those tested. Some characteristics of the reduction of superoxide dismutase by $NaBH_4$ were further investigated. The results obtained indicate that $NaBH_4$ can be used to prepare, in a few minutes, solutions of completely reduced enzyme without any apparent change of the activity and of the structure.

The Cu,Zn superoxide dismutase (EC 1.15.1.1) has been extensively studied in recent years since the discovery of its ability to catalyse the dismutation of superoxide ion, $O_2^{\cdot-}$ (McCord & Fridovich, 1969). During the catalytic cycle the copper in the active site is alternately reduced and oxidized according to the scheme:



where $k_1 = k_2 = 2.3 \times 10^9 M^{-1} \cdot s^{-1}$ (Fielden *et al.*, 1974). Since the kinetic rate constants are equal, half of the enzyme copper is present as Cu^+ in systems where $O_2^{\cdot-}$ is continuously generated, such as in pulse-radiolysis experiments in aqueous solutions (Fielden *et al.*, 1974; Cockle & Bray, 1977), or in erythrocytes (Scarpa *et al.*, 1984*a*,*b*). However, in comparison with the great number of papers on the oxidized superoxide dismutase, very few studies have been carried out on the reduced enzyme. This is due mainly to the fact that Cu^+ is silent in the usual spectroscopic methods and therefore it cannot be utilized as a probe of the active site. Furthermore, the easy re-oxidation of the reduced enzyme by molecular O_2 and the absence of a simple and well-characterized method for the reduction of superoxide dismutase may have contributed to the lack of studies on the reduced enzyme. In fact, the chemicals most frequently utilized to reduce superoxide dismutase, i.e. dithionite and H_2O_2, are potentially inactivating agents. The aim of the present paper is the comparison of different reducing agents on the basis of their ability to reduce the enzymic Cu^{2+} to Cu^+ and of the lack of enzyme inactivation. Since $NaBH_4$, apart from being the most convenient reducing agent among those tested, does not change the structure and the activity of the enzyme, we further investigated the characteristics of the enzyme reduction.

Materials and methods

Bovine Cu,Zn superoxide dismutase was prepared from bovine erythrocytes by the method of McCord & Fridovich (1969). All solutions were prepared from analytical-grade chemicals. $NaBH_4$ was purchased from Fluka, and solutions were prepared just before use by dissolving the required amount of $NaBH_4$ in 0.1 M-sodium borate buffer, pH 10.2, to give 0.1 M-$NaBH_4$.

^1H- and $^{19}F^-$-nuclear-magnetic-relaxation measurements were carried out with a pulse n.m.r. apparatus operating at 16 MHz. T_1 was measured by using the $\pi/2$–τ–$\pi/2$ sequence (Martin *et al.*, 1980).

The superoxide dismutase activity measurements were carried out as previously described (Rigo *et al.*, 1975), with an Amel model 461 polarographic unit. Polyacrylamide-gel electrophoresis was carried out as described by Rigo

et al. (1977); X-band low-temperature e.p.r. spectra were obtained with a Brucker ER 200 D spectrometer.

Results and discussion

H_2O_2, $NaBH_4$, ferrocyanide and dithionite as reducing agents of Cu,Zn superoxide dismutase

The reduction of the Cu^{2+} present in the active site of Cu,Zn superoxide dismutase to Cu^+ determines the bleaching of the e.p.r. spectrum and of the 660nm absorption band that characterize the oxidized enzyme. Furthermore, in aqueous solutions the reduction process is accompanied by the decrease of the nuclear magnetic relaxation rate (T_1^{-1}) of the water protons and of ^{19}F to values very close to that observed in the absence of superoxide dismutase.

The n.m.r. method appears to be particularly suitable for monitoring the enzyme reduction, since the measurements can be carried out at room temperature and are unaffected by the presence of the reducing agent or by the small bubbles that are formed when H_2O_2 or $NaBH_4$ is used as reducing agent and, more importantly, the paramagnetic contribution to the relaxation rate of ^1H and of $^{19}F^-$ (T_{1p}^{-1}) is proportional to the concentration of ECu^{2+} (Terenzi *et al.*, 1974; Viglino *et al.*, 1979). With the use of the n.m.r. method, H_2O_2, dithionite, $NaBH_4$ and ferrocyanide were tested for their ability to reduce the superoxide dismutase, and the activity and the electrophoretic pattern of the enzyme treated with the different reducing agents were also checked. The results obtained (Table 1) show that the enzyme is almost completely reduced by dithionite and by $NaBH_4$, whereas a non-negligible amount of ECu^{2+} was still present after reduction with H_2O_2 and ferrocyanide. In the case of H_2O_2 a similar result was also obtained by Bray *et al.* (1974). Under our experimental conditions, the enzyme reduced by $NaBH_4$ or by ferrocyanide retained full activity, whereas a strong decrease of the activity, about 70%, was observed with H_2O_2 and dithionite. Polyacrylamide-gel electrophoresis of the superoxide dismutase treated with the different reducing agents and re-oxidized by molecular O_2, after removal of the reducing agent, was in agreement with the activity measurements. In fact, the $NaBH_4$- or ferrocyanide-reduced enzyme showed electrophoretic behaviour identical with that of native superoxide dismutase. In contrast, the gels of the enzyme treated with dithionite or H_2O_2 presented a pattern of intense bands with higher electrophoretic mobility than that of native superoxide dismutase, indicating an extensive inactivation of the enzyme. On this basis it appears that only $NaBH_4$ and ferrocyanide can be considered as safe reducing agents of the enzyme, though the latter reagent appears to be a weaker reductant than $NaBH_4$. The possibility of eliminating the excess of reducing agent, and the reduction by-products, should be considered when accurate studies must be carried out on the reduced enzyme. This is not a problem when $NaBH_4$ or H_2O_2 is utilized as reductant, since the addition of a very weak acid (e.g. acetic acid, KH_2PO_4 etc.) or of catalase respectively determines a fast and complete transformation of the reagent excess to H_3BO_3 or O_2 respectively, which are normal components of buffer systems. In contrast, for ferrocyanide and dithionite the removal of their excess and of the reaction by-products requires exclusion chromatography or dialysis that must be carried out under anaerobic conditions to avoid the re-oxidation of the reduced enzyme. In fact, it should be noted that the oxidation by molecular O_2 of the fully reduced superoxide dismutase to about a half-oxidized state, which is a complex process, occurs in a few minutes at physiological pH values.

$NaBH_4$ as a reducing agent of superoxide dismutase

The time course of the reduction of superoxide dismutase with $NaBH_4$ was followed under pseudo-first-order conditions, i.e. with $[NaBH_4]/[\text{superoxide dismutase}] \geqslant 10$ in 0.1 M-sodium borate buffer, pH 10, by measuring the T_1 of the water protons of the reaction system. Under these conditions the reduction follows first-order kinetics with respect to the oxidized enzyme, i.e. the plot

Table 1. *Reduction level and activity of Cu,Zn superoxide dismutase treated with different reducing agents*
In all the experiments the superoxide dismutase concentration was 1 mM; the reductant concentration, the pH value and the reduction time were as follows: ferrocyanide, 1 mM, pH 7.4, 30 min; $NaBH_4$, 3 mM, pH 10, 8 min; dithionite, 5 mM, pH 7.4, 2 min; H_2O_2, 3 mM, pH 9.2, 15 min. The ECu^{2+} content was measured by $^{19}F^-$ n.m.r, by the addition of a concentrated solution of F^- to the reduced enzyme solution (Viglino *et al.*, 1979).

Reducing agent	Residual ECu^{2+} (%)	Activity of the reduced enzyme (%)
$K_4Fe(CN)_6$	12.2	103
$NaBH_4$	3.0	98
$Na_2S_2O_4$	1.0	32
H_2O_2	23.0	33

of $\ln([ECu^{2+}]_0/[ECu^{2+}])$ versus time, where $[ECu^{2+}]_0$ is the initial concentration of ECu^{2+}, is a straight line (see Fig. 1).

Furthermore, since (under the conditions reported above and in the range 1–10mM-$NaBH_4$) the pseudo-first-order kinetic constant, k', is linearly dependent on $NaBH_4$ concentration (see Fig. 2), the reduction process is also first-order with respect to $NaBH_4$. Therefore we can write:

$$\ln\left(\frac{[ECu^{2+}]_0}{[ECu^{2+}]}\right) = k[NaBH_4]t \qquad (1)$$

and $k = 4.8 \times 10^2 M^{-1} \cdot min^{-1}$ can be calculated from the slope of Fig. 2. The degree of enzyme reduction can be easily calculated from eqn. (1), and it appears that with $[NaBH_4] \geqslant 3$mM the reduction of ECu^{2+} to ECu^{+} is practically quantitative within a few minutes. It must be stated that a small fraction of Cu^{2+}, 2–5% depending on the enzyme batch, was usually detected, both by T_1 and by low-temperature e.p.r. measurements, after 30min of reduction with 10mM-$NaBH_4$ under anaerobic conditions. It seems reasonable to ascribe this small fraction of non-reducible copper to denaturated enzyme molecules always present in the superoxide dismutase preparations. To stop the reduction, the reaction system was usually adjusted to pH4–5 by addition of a calculated amount of 5M-acetic acid. Under these conditions the $NaBH_4$ is converted almost instantaneously into H_3BO_3 and H_2. As regards possible modifications of the enzyme by $NaBH_4$, the activity, the e.p.r. spectrum and the thiol content of superoxide dismutase were studied before and after reduction. In particular, a study of the dependence of superoxide dismutase activity on the reduction time showed that the enzyme retains the full activity within 10min from the beginning of the reduction. It should be noted that, within this time, operating under the conditions reported above, the enzyme is completely reduced. However, with reaction times longer than 10min, the enzyme activity decreased, as reported in Fig. 3. From this Figure it appears that after 1h the enzyme activity is halved and after 3h has decreased to about 25%. This decrease of the activity cannot be ascribed to a direct modification of the enzyme by $NaBH_4$. In fact, when the reduction was carried out under anaerobic conditions the enzyme retained full activity after 1h of incubation with 10mM-$NaBH_4$ at pH10. As a consequence it results that O_2, or its reduction products, are involved in the enzyme deactivation under aerobic conditions. In particular, H_2O_2 appears the most likely candidate for the observed inactivation of superoxide dismutase on account of the denaturing properties of H_2O_2 (Bray *et al.*, 1974; Hodgson & Fridovich, 1975) and of its formation during the oxidation of the enzyme

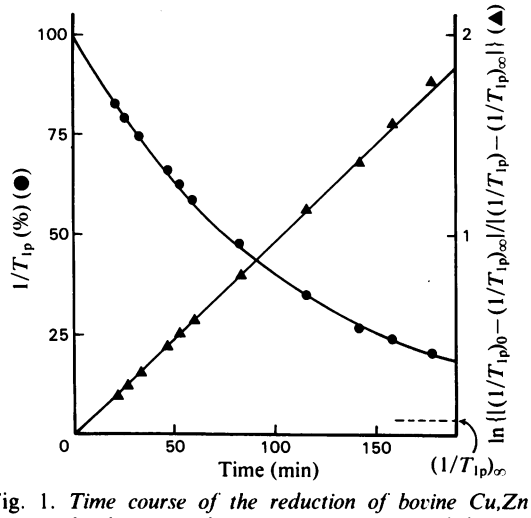


Fig. 1. *Time course of the reduction of bovine Cu,Zn superoxide dismutase by $NaBH_4$ as measured by the paramagnetic contribution to the relaxation rate of water protons (T_{1p}^{-1}) (●)*

The straight line (▲) is the corresponding first-order plot. The reduction medium contained 0.2mM-superoxide dismutase and 3.4mM-$NaBH_4$ in 0.1M-borate buffer, pH10. The temperature was 22°C.

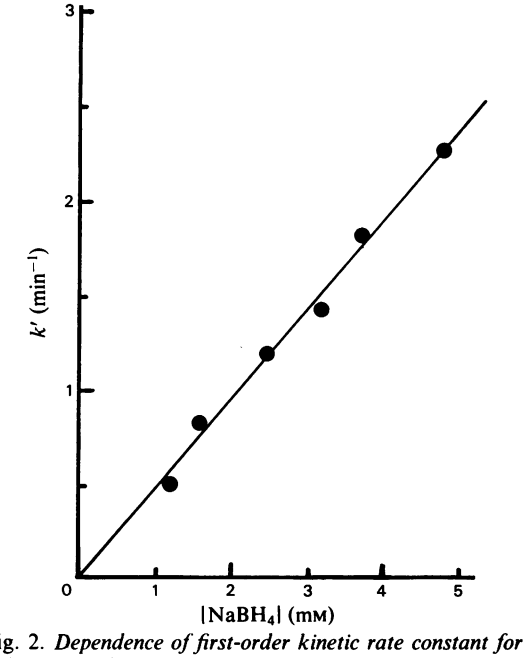


Fig. 2. *Dependence of first-order kinetic rate constant for reduction of bovine Cu,Zn superoxide dismutase on the $NaBH_4$ concentration*

The enzyme concentration was 0.2mM. All measurements were carried out in 0.1M-borate buffer, pH10, at $[NaBH_4]/[\text{superoxide dismutase}] \geqslant 10$.

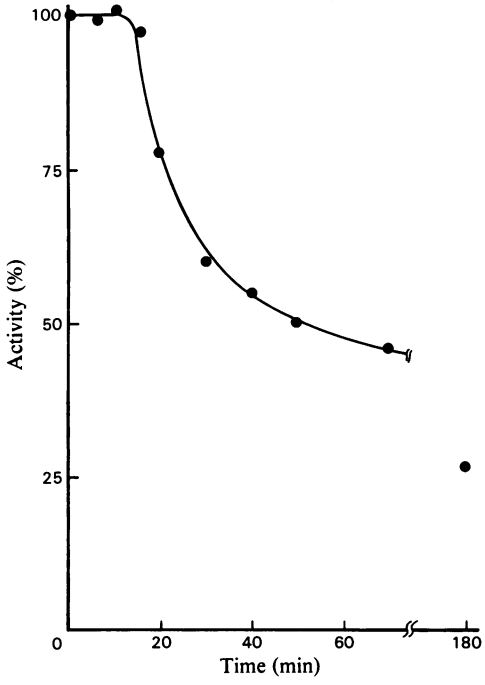


Fig. 3. *Inactivation of bovine Cu,Zn superoxide dismutase by $NaBH_4$*
For conditions see the legend to Table 1.

by molecular O_2, which occurs very quickly at pH 10 (P. Viglino, M. Scarpa, G. Rotilio & A. Rigo, unpublished work). The mildness of the $NaBH_4$ reduction was confirmed by the e.p.r. spectrum of re-oxidized enzyme, which showed no significative difference from that of the native enzyme, indicating that the typical copper co-ordination of the native superoxide dismutase is not affected by $NaBH_4$ reduction. In this case the re-oxidation of the enzyme was carried out by molecular O_2 at pH 8 in the presence of catalase to eliminate the H_2O_2 possibly formed.

Since each superoxide dismutase molecule contains in addition to the two free thiol groups two intersubunit disulphide bonds (Abernethy *et al.*, 1974), we investigated the possible reduction by $NaBH_4$ of the intersubunit disulphide bond. The thiol titration was carried out with 5,5′-dithiobis-(2-nitrobenzoic acid) by the procedure of Habeeb (1972). Both the native enzyme and the enzyme treated with 10mM-$NaBH_4$ for 1 h were incubated with 0.5mM-5,5′-dithiobis-(2-nitrobenzoic acid) in 6M-guanidine in phosphate buffer, pH 6.8. The thiol content was 2.10 and 2.02 thiol groups/enzyme molecule respectively, indicating that the disulphide group is not reduced by $NaBH_4$, probably because the group is well-buried inside the enzyme molecule.

In conclusion, the results presented here indicate that superoxide dismutase can be easily and safely reduced by $NaBH_4$, whereas other reductants, such as dithionite and H_2O_2, which in past years have been largely used for reduction of superoxide dismutase, partially denatured the enzyme.

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