Biochimica et Biophysica Acta, 566 (1979) 32—39

BBA 68630

EVALUATION OF NEGATIVE STAINING TECHNIQUE FOR DETERMINATION OF CN^--INSENSITIVE SUPEROXIDE DISMUTASE ACTIVITY

GUGLIELMO DE ROSA *, DAVID S. DUNCAN, CARL L. KEEN and LUCILLE S. HURLEY

Department of Nutrition, University of California, Davis, CA 95616 (U.S.A.)

(Received April 14th, 1978)
(Revised manuscript received August 22nd, 1978)

Key words: CN^--insensitive; Superoxide dismutase; Negative staining; (Isoenzyme)

Summary

The different forms of superoxide dismutase (superoxide-superoxide oxidoreductase, EC 1.15.1.1) have been studied, in tissues of rat, mouse and chicken, by the electrophoresis-nitro blue tetrazolium technique proposed by Beauchamp. Similar enzyme patterns were evident in every tissue. A fast migrating CN^--sensitive form of dismutase activity was present in isolated liver mitochondria of each species. Chicken and mouse liver mitochondria, as well as whole homogenate of every tissue of these two species, showed two additional slow-migrating bands of CN^--insensitive activity. In contrast, such bands were not detectable in mitochondria isolated from rat liver or in any of the rat tissues analyzed by this technique.

Prior to their electrophoretic separation, the samples were analyzed for CN^--insensitive superoxide dismutase activity by a spectrophotometric assay; by this assay it was possible to demonstrate and quantitate a CN^--insensitive superoxide dismutase activity in every preparation. Two units of CN^--insensitive activity were applied to the gels for each sample. These results indicate that the electrophoresis-nitro blue tetrazolium technique is unsuitable for the detection of the rat CN^--insensitive form of superoxide dismutase in crude preparations such as whole tissue homogenates or isolated mitochondria.

Introduction

Superoxide dismutase (superoxide-superoxide oxidoreductase, EC 1.15.1.1) catalyzes the disproportionation or dismutation of superoxide free radicals by

* Present address: Digestive Diseases and Nutrition Section, School of Medicine, Department of Medicine, University of Louisville, P.O. Box 35260, Louisville, Ky. 40232; and to whom reprint requests should be addressed.

the following reaction:

$$O_2^- + O_2^- + 2H \rightarrow O_2 + H_2O_2$$

The enzyme was discovered and isolated from bovine erythrocytes in 1969 [1]. The recognition of its enzymic activity came from the observation that a protein existed which was a competitive inhibitor of the reduction of cytochrome *c* by xanthine oxidase [2]. Further studies have demonstrated that superoxide dismutase acts an inhibitor in both reducing and oxidizing reactions dependent on the presence of free O_2^- [3]. This property has been used to assay the dismutase activity.

The enzyme thus far has been demonstrated to exist in different forms in every aerobic organism [4]. In prokaryotes two superoxide dismutases have been isolated: (1) an iron-enzyme localized in the periplasmic space and (2) a mangano-enzyme in the cell matrix [5,6]. Eukaryotic cells have a copper-enzyme in the cytoplasm, which also contains Zn^{2+}; in the mitochondrial matrix a Mn^{2+}-enzyme is present which, on the basis of its amino acid composition, appears to be closely related to the Mn^{2+}-superoxide dismutase found in prokaryotes [7].

Several forms of superoxide dismutase can be differentiated by coupling their electrophoretic separation with the colorimetric assay using nitro blue tetrazolium [8]. Weisiger and Fridovich [9] have reported the presence of four fast-migrating bands of superoxide dismutase activity and three more slowly migrating ones in *Saccharomyces cerevisiae* and in chick liver. The first group of fast-migrating bands represents the cupro-zinc type of superoxide dismutase, which is sensitive to inhibition by CN^-; the slow-migrating bands, on the other hand, are formed by the Mn^{2+}-enzyme, insensitive to CN^- inhibition.

This method has also allowed these authors to determine, in isolated mitochondria of *S. cerevisiae* and chicken liver, the presence of two CN^--sensitive forms of superoxide dismutase migrating at the same rate as the cupro-zinc cytosolic type of the enzyme. These two forms appear to be localized in the intermembrane space of the mitochondria [9].

The high sensitivity of this technique has been used by several investigators to demonstrate the presence of the various forms of superoxide dismutase in mouse and human liver [10,11]. The same laboratories, on the other hand, have reported the inability to detect any CN^--insensitive superoxide dismutase in some tumors as well as in rat liver by this technique. However, this form of the enzyme was found to be present in other rat tissues when its activity was determined by a spectrophotometric assay in which the oxidation of xanthine by xanthine oxidase was used as the source of free O_2^- [12,13].

Because of the possible primary role of the mitochondrial enzyme in scavenging free O_2^- produced by cellular respiration, we were interested in clarifying whether the rat Mn^{2+}-superoxide dismutase is an enzyme different from that found in mouse and chicken liver; this enzyme might be lacking or inhibited under ordinary conditions in rat liver or in other tissues of this species. Alternatively, perhaps in Mn^{2+}-enzyme cannot be detected in the rat by methods which can be used to determine the CN^--insensitive activity in other species.

The data obtained in this investigation indicate that a CN^--insensitive super-

oxide dismutase is present in rat tissue, but it cannot be detected when crude preparations are analyzed by the technique proposed by Beauchamp.

Materials and Methods

Male albino rats (Sprague-Dawley, 100—150 g), mice (four way cross of C57B1/6J; C3H/J; AKR/J and DBA/2J, 35—40 g) and white Leghorn chickens were used. All animals had been raised on stock diets (Purina Rat Chow; Purina Laboratory Chow (fed to mice); Purina Starter Chick Ration, Ralston Purina Co., St. Louis, Mo.). On the day of the experiment the animals were anesthetized with diethyl ether, and saline solution was perfused into the right atrium until the extremities appeared pale. Liver, heart, brain, and lung were excised and placed in cold (4°C) medium of the following composition: 0.220 M mannitol, 0.07 M sucrose, 0.002 M HEPES (pH 7.8). The tissues were blotted dry, weighed and homogenized in 3 vols. phosphate buffer (pH 7.8), 10^{-3} M EDTA, using a Potter-Elvejhem homogenizer with a tight-fitting pestle. Part of the liver was homogenized in 3 vols. mannitol/sucrose/HEPES medium using a glass homogenizer with a loose-fitting Teflon pestle. All homogenates were centrifuged at $750 \times g$ for 10 min. Liver mitochondria were isolated by the method of Schnaitman and Greenawalt [15]; the final mitochondrial pellet was resuspended in phosphate buffer (pH 7.8), 10^{-3} M EDTA, in 1/4 of the original homogenate volume.

The mitochondrial preparations and homogenates were subjected to 2 cycles of freeze-thawing, then sonicated for 2 min (30 s with 30 s cooling) with an Insonator Model 500 (Savant Instruments, Inc., Hicksville, N.Y.) set at maximum power. The samples were then centrifuged at $105\,000 \times g$ for 45 min. The pellets were discarded and the supernatant fractions immediately assayed for superoxide dismutase activity by the method of McCord and Fridovich [1] which is based on the inhibition of epinephrine oxidation rate by O_2^- released by the action of xanthine oxidase on xanthine at pH 7.8. The activity of the mitochondrial enzyme was determined by repeating the assays in the presence of 2 mM NaCN. Aliquots of the samples were analyzed by polyacrylamide gel electrophoresis [16]; the gels (0.5×13 cm) were negatively stained for superoxide dismutase by the riboflavin-nitro blue tetrazolium method [8]. After staining, the gels were stored in $CH_3OH/CH_3COOH/H_2O$ (49 : 1 : 50, v/v).

Protein was determined by the method of Lowry et al. [17]. All reagents were of the purest grade available from Sigma Chemical Co., St. Louis, Mo. Reagents for electrophoresis were obtained from Bio-Rad Laboratories, Richmond, Calif.

Results

The activity of the CN^--insensitive superoxide dismutase (assayed in the presence of 2 mM NaCN) in liver mitochondria, as well as in homogenates of mouse and rat liver, brain, heart and lung, are summarized in Table I. Total dismutase activity has not been reported because the variability in the amount of blood trapped in each tissue made the obtained values inconsistent and, therefore, unreliable, in spite of extensive perfusion of the animals with saline. CN^--

TABLE I

CN⁻-INSENSITIVE SUPEROXIDE DISMUTASE ACTIVITY IN RAT AND MOUSE TISSUES

One unit of activity defined as the amount of enzyme causing 50% inhibition of the oxidation of epinephrine at 30°C.

Tissue	Species	
	Rat	Mouse
Brain *	193 ± 17	125 ± 6.5
Heart	197 ± 28	165 ± 6.1
Liver	500 ± 79	186 ± 18
Lung	32 ± 2	71 ± 3.3
Liver mitochondria **	9.55 ± 0.36	4.69 ± 0.23

* Units/g tissue wet weight ± S.E. of at least four animals.
** Units/mg protein ± S.E. of at least four animals.

insensitive enzyme activity was present in each of these tissues, with the highest activity found in the liver. As expected, no superoxide dismutase activity could be detected in whole blood when the assay was performed in the presence of 2 mM NaCN. The lowest tissue activity was always observed in lung in both species, in spite of the fact that this tissue was the most difficult to obtain free of blood contamination by our perfusion technique.

As we were interested in accurately determining the mitochondrial dismutase activity, we wanted to test the completeness of the CN^- inhibition in our system. Fig. 1 shows the enzyme activity in both total liver homogenate and isolated rat liver mitochondria in the presence of increasing amounts of CN^- in the reaction medium. Two units of total superoxide dismutase activity were used for both samples. The results are expressed as percentage inhibition of the epi-

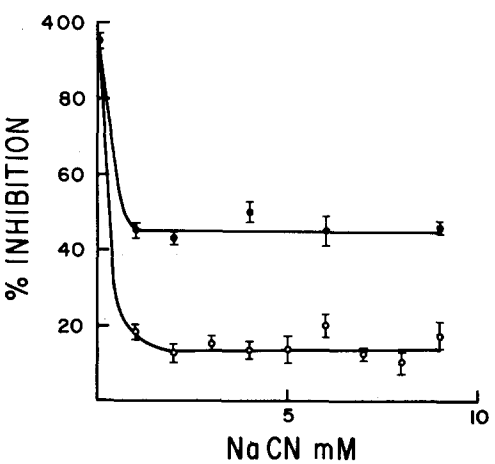

Fig. 1. Inhibition of epinephrine oxidation rates by 105 000 × *g* supernatant fractions of rat liver homogenate (○) and isolated sonicated rat liver mitochondria (●) in presence of increasing concentrations of NaCN. The assays were performed at 30°C in $2 \cdot 10^{-4}$ M epinephrine, $1 \cdot 10^{-4}$ M xanthine and $1 \cdot 10^{-8}$ M milk xanthine oxidase (Sigma grade IV), NaCN at the indicated concentrations, in 0.05 M phosphate buffer, 10^{-3} M EDTA (pH 7.8). Each point represents the mean of at least four determinations; the S.E. is indicated by the vertical bars.

nephrine oxidation obtained at that particular CN^- concentration in the absence of the dismutase containing sample. The maximal inhibitory effect was obtained for both preparations between 1 and 2 mM NaCN; no further decrease in the inhibition of epinephrine oxidation was obtained by increasing the NaCN concentration up to 8 mM.

The assay developed by Beauchamp [8] was used to compare the patterns of superoxide dismutase activities detectable after polyacrylamide gel electrophoresis of mitochondrial preparations of chicken, mouse and rat liver. Two units of CN^--insensitive activity, as determined by the spectrophotometric assay were applied to the gels. In the mitochondria of the three species, a broad, fast-migrating band of activity was present, which disappeared when the gels were stained in the presence of 2 mM NaCN (Fig. 2). Two slow-migrating forms of CN^--insensitive enzyme activity were present in mouse and chicken liver mitochondria, which could not be detected in a similar preparation from rat liver. Similar results were obtained by increasing the pH of the Tris-glycine buffer to 9.5, or by lowering the acrylamide content of the gels to 5%.

In a number of instances [18—20] isozyme patterns have been shown to vary from tissue to tissue in the same species and among different species. We were, therefore, interested in determining whether the differences observed between the CN^--insensitive superoxide dismutase of rat, mouse and chicken liver were restricted to this tissues or whether they reflected a specific difference in this enzyme among species.

The pattern of dismutase forms in whole homogenates of liver, lung, brain and heart of rat, mouse and chicken was compared by polyacrylamide gel electrophoresis. Two units of CN^--insensitive enzyme were applied for each sample, with the exception of lung tissue; the dismutase specific activity is very low in this tissue and aliquots containing more than one unit of the enzyme could not be applied without overloading the gels with protein. In every tissue, a fast-migrating band of activity was clearly evident, which could not be detected when 2 mM NaCN were added to the reaction medium (Fig. 3). In each tissue from mouse and chicken, two slow-migrating bands of CN^--insensitive enzyme were detectable. This was also the case with lung preparations, even though the

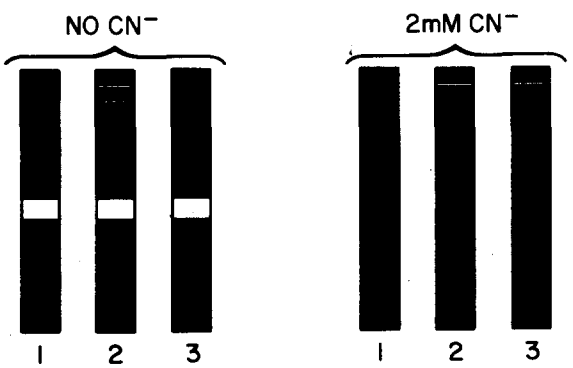

Fig. 2. Polyacrylamide gel electrophoresis of rat (1), mouse (2), and chicken (3) isolated liver mitochondria. The gels, 7.5% acrylamide, were stained for superoxide dismutase in the presence (right panel) or absence (left panel) of 2 mM NaCN.

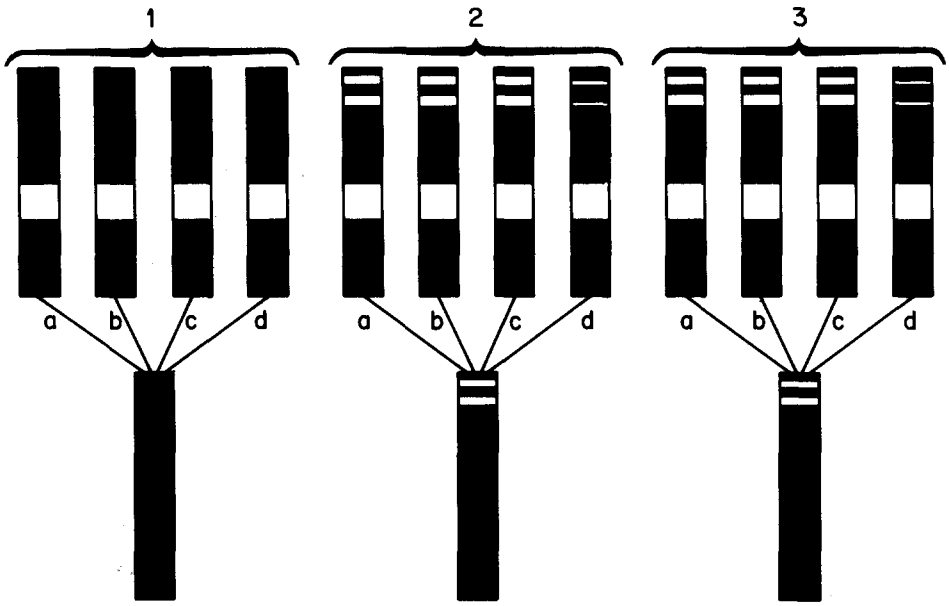

Fig. 3. Polyacrylamide gel electrophoresis of 105 000 X *g* supernatant fractions from brain (a), heart (b), liver (c) and lung (d) homogenates of rat (1), mouse (2) and chicken (3). Upper panel, no NaCN present in the staining solutions. Lower panel, 2 mM NaCN added to the staining solutions. Aliquots of the homogenates containing 2 units of CN^--insensitive superoxide dismutase activity were applied for each sample with the exception of the lung (d), where 1 unit of CN^--insensitive enzyme was applied for all species.

amount of enzyme applied was only 1/2 of that used for other tissues. No detectable superoxide dismutase activity was seen in any of the gels containing rat tissue homogenates when the gels were stained in the presence of NaCN.

Discussion

Superoxide dismutase isoenzymes can be studied by coupling their electrophoretic separation with their ability to inhibit the formation of the formazan derivative of nitro blue tetrazolium. The main advantage of this method is that in only one step it is possible to determine the enzyme activity in crude preparations, separated from disturbing factors which might interfere with the enzymatic reaction. This feature, together with the reported high sensitivity for the cytosolic enzyme activity [21], allows the study of the different forms of dismutase on samples of limited size, with only limited loss of enzyme activity. It has thus been possible to separate the different forms of the CN^--sensitive and CN^--insensitive groups of dismutase activity, as well as the cytosolic and the mitochondrial ones.

In the present investigation, we applied this method to compare the different forms of superoxide dismutase in mouse, chicken and rat tissues. In agreement with other reports we have demonstrated the presence of the CN^--insensitive enzyme, together with the CN^--sensitive group, in mouse and chicken liver. The pattern of electrophoretically separated forms of the dismutase was very similar from one tissue to another in mouse and chicken. In mitochondria isolated

from rat liver, as well as from mouse and chicken liver, a broad band of CN^--sensitive dismutase activity was found, with a migration rate equal to the cytosolic Cu^{2+}/Zn^{2+}-enzyme. In addition to this CN^--sensitive form, in each tissue from chicken and mouse, two CN^--insensitive forms of superoxide dismutase are present; these forms represent the mitochondrial Mn^{2+}-enzyme first described by Weisiger and Fridovich in chicken liver [9].

The electrophoretic technique applied to preparations from rat liver did not reveal any CN^--insensitive dismutase activity in either isolated mitochondria or total tissue homogenates, in agreement with others [22]. This is, however, in conflict with the results obtained by spectrophotometric assay of the enzyme. We were able to detect in each rat tissue some CN^--insensitive dismutase activity, with a distribution similar to that observed in the other species analyzed (that is, highest activity in liver, lowest in lung). Other laboratories have reported the presence of a CN^--insensitive dismutase activity in some rat tissues; in each case, a spectrophotometric assay similar to the one in this study was used.

The validity of our method for the determination of the CN^--insensitive mitochondrial superoxide dismutase activity is indicated by the following considerations:

(1) It has been reported that 1 mM NaCN completely inhibits the Cu^{2+}/Zn^{2+} form of the enzyme, without affecting the activity of the mitochondrial Mn^{2+}-enzyme. In our system, we used 2 mM NaCN and, in fact, at this concentration, we obtained complete inhibition of the CN^--sensitive enzyme; CN^- did not affect the mitochondrial activity, even at concentrations as high as 8 mM.

(2) The activity of the cytosolic enzyme was completely inhibited in our system, as demonstrated by the observations that whole blood had no detectable dismutase activity and that lung (which was the tissue most contaminated by blood) had consistently the lowest enzyme activity.

Van Berkel et al. [23], using the electrophoretic technique, reported an inability to detect any CN^--insensitive dismutase activity in whole rat liver preparations. Yet, after separation of parenchymal from non-parenchymal liver cells, a CN^--insensitive band of enzymatic activity was present when the parenchymal cells were analyzed by this technique. As no inhibition of the dismutase activity could be obtained by recombining the parenchymal with non-parenchymal cells, these authors advanced the hypothesis that an activation of the enzyme had occurred in the course of the isolation of the two kind of cells. Our investigation cannot offer an explanation for this enzyme activation postulate. It, instead, provides some possible explanations for the conflicting reports on the existence of a CN^--insensitive superoxide dismutase in rat tissues.

The results reported by Van Berkel et al. [23] clearly indicate that the preparation of rat liver parenchymal cells had a dismutase activity which was 1.5 and 2 times higher than that of the whole rat liver and of the non-parenchymal cells, respectively. These authors did not indicate how much protein or enzyme activity was applied to the gels for each sample. If, in comparing parenchymal cells and whole liver homogenate, equal amounts of protein or of total dismutase activity were applied to the gels, the amount of mitochondrial activity applied would have been almost twice as high for the parenchymal cell as for

the other samples. In this case, their results indicate that whole liver samples analyzed by electrophoresis contain amounts of dismutase which are below the threshold of sensitivity of the method employed.

In the present investigation, an amount of each sample equal to two units of spectrophotometrically-determined CN^--insensitive superoxide dismutase activity was applied to the gels. The difference in results obtained by the spectrophotometric assay involving epinephrine oxidation and those obtained by the Beauchamp technique can be explained by assuming that the rat mitochondrial CN^--insensitive enzyme has an affinity for O_2^- which is lower than that of the chicken, mouse and human form of dismutase and, further, that the concentration of free O_2^- obtained by riboflavin activation is lower than that obtained by xanthine oxidation.

Another possibility which might be also considered is that the rat CN^--insensitive dismutase, in the 'inactive' form, does not penetrate the gels and that it is somewhat modified upon 'activation' to a form which does penetrates the gels and can be assayed by this method. Our results appear to eliminate almost completely such a possibility. By increasing the pH of the reservoir buffers and decreasing the percentage of acrylamide used for the gels, it was still not possible to detect any CN^--insensitive dismutase activity in any rat tissues, while it was always possible to detect this form in tissues of the other species studied.

Acknowledgment

This investigation was supported in part by NIH Research Grant No. HD-02355 from the National Institute of Child Health and Human Development and National Research Service Award DE-07001 from the National Institute of Dental Research.

References

1 McCord, J.M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6049—6055
2 Fridovich, I. (1967) J. Biol. Chem. 242, 1445—1450
3 McCord, J.M. and Fridovich, I. (1969) J. Biol. Chem. 244, 6056—6063
4 Fridovich, I. (1974) Adv. Enzymol. 41, 35—97
5 Gregory, E.M., Yost, F.J. and Fridovich, I. (1973) J. Bacteriol. 115, 987—991
6 Yost, F.J. and Fridovich, I. (1973) J. Biol. Chem. 248, 4905—4908
7 Weisiger, R.A. and Fridovich, I. (1973) J. Biol. Chem. 248, 3582—3592
8 Beauchamp, C. and Fridovich, I. (1971) Anal. Biochem. 44, 276—280
9 Weisiger, R.A. and Fridovich, I. (1973) J. Biol. Chem. 248, 4793—4796
10 Sahu, S.K., Oberley, L.W., Stevens, R.H. and Riley, E.F. (1977) J. Natl. Cancer Inst. 58, 1125—1128
11 Salin, M.L. and McCord, J.M. (1974) J. Clin. Invest. 54, 1005—1009
12 Watson, R.R., Rister, M. and Boehner, R.L. (1976) J. Nutr. 103, 1801—1808
13 Tyler, D. (1975) Biochem. J. 147, 493—504
14 Fridovich, I. (1975) Annu. Rev. Biochem. 44, 147—159
15 Schnaitman, C. and Greenawalt, J.W. (1968) J. Cell Biol. 38, 158—164
16 Davis, B.J. (1964) Ann. New York Acad. Sci. 121, 404—427
17 Lowry, O.H., Rosebrough, N., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
18 Appella, E. and Markert, C.L. (1961) Biochem. Biophys. Res. Commun. 6, 171—177
19 McFarlane, I.G. and Von Holt, C. (1969) Biochem. J. 111, 557—565
20 deRosa, G. and Swick, R.W. (1975) J. Biol. Chem. 250, 7961—7967
21 Bohnenkamp, W. and Weser, U. (1975) Hoppe-Seyler's Z. Physiol. Chem. 356, 747—754
22 Ntzumi, K., Yoshioka, T., Yamanaka, N. and Nakazawa, T. (1977) FEBS Lett. 79, 1—3
23 Van Berkel, T.J.C., Kruijt, J.K., Slee, R.G. and Koster, J.F. (1977) Arch. Biochem. Biophys. 179, 1—7