Vol. 125, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1984 Pages 425-430

COPPER FACILITATED CHEMILUMINESCENCE FROM THE SULFHYDRYL PROTEINS: YEAST ALCOHOL DEHYDROGENASE, SPINACH FERREDOXIN AND METALLOTHIONEIN

Ka-Ho Wong[+], Erin E. Boh[++] and Richard H. Steele[++,+++]

Departments of Pharmacology[+] and Biochemistry[++,+++],
Tulane University School of Medicine, New Orleans, LA 70112

Received October 26, 1984

SUMMARY: This paper describes a copper mediated formation of active oxygen, presumably $O_2^{\cdot-}$, from the sulfhydryl proteins, yeast alcohol dehydrogenase spinach ferredoxin and rabbit liver metallothionein which, on the addition of cyanide in the presence of acetaldehyde, displays as a chemiluminescence. These studies may provide some insights into the mechanisms of copper toxicity and provide a sensitive assay for monitoring the presence of sulfhydryl groups.

Chemiluminescence reflects the emissive relaxation of high energy electronic excitation states populated in general by the exothermic reactions of oxygen peroxides, oxygen radicals or singlet oxygen. Chemiluminescence, therefore, is a sensitive and useful phenomenon for monitoring the generation and fate of active oxygen species.

Boh et. al. (1) reported an acetaldehyde-dependent chemiluminescence from mitochondria. The intensities and kinetics of the emissions responded with exquisite sensitivity to substrates, ADP, ATP, pO_2 and the site blockers and inhibitors rotenone, antimycin A, malonate, azide and cyanide.

Unexpectedly, the cyanide-evokable acetaldehyde-dependent chemiluminescence was elicitable from heated, 90°C for 10 min., mitochondria and submitochondrial particles. We reasoned that a heat-resistant source of reducing equivalents must be supporting the light and considered sulfhydryl moieties within the redox chain as likely candidates. This thesis was supported by our findings that emission responses were attenuated progressively by increasing concentrations of N-ethylmaleimide. Subsequently, Boh and Steele (2) were

+++To whom correspondence should be addressed.

0006-291X/84 $1.50

able to evoke an acetaldehyde-dependent chemiluminescence from the sulfhydryl containing proteins, yeast alcohol dehydrogenase and spinach ferredoxin.

Shortly after the above studies were completed we lost the ability to elicit the cyanide-evokable acetaldehyde-dependent chemiluminescence from both the mitochondria and the sulfhydryl proteins. After eight frustrating months we discovered the requirement for copper (3). The addition of trace amounts of copper restored the cyanide-evokable acetaldehyde-dependent emissions from both, mitochondria and the sulfhydryl containing proteins. In this paper we present the copper mediated chemiluminescene data, plotted for kinetic evaluation, for yeast alcohol dehydrogenase, spinach ferredoxin and rabbit liver metallothionein.

MATERIALS AND METHODS

Yeast alcohol dehydrogenase (E.C.1.1.1.1.), spinach ferredoxin, isolated according to Tagawa and Arnon (4), and rabbit liver metallothionein were obtained from Sigma. Distilled and/or deionized water was redistilled in an all glass still. All chemicals were reagent grade. In each experiment chemiluminescence was initiated by the terminal addition of cyanide. Chemiluminescent measurements were made as previously described (1). The method for the evaluation of the series first-order rate constants was as described previously (5). The integral chemiluminescence, total counts under the curve ($h\nu_I$), was determined by dividing A_0 by k_2, where A_o, the concentration of A at zero-time, was determined by extrapolating the linear portions of the semilog plots for the $B \rightarrow C + h\nu$ decay back to zero-time.

RESULTS AND DISCUSSION

We present in Fig. 1, semilog plots of the cyanide-evokable, copper-acetaldehyde-dependent chemiluminescence versus time for yeast alcohol dehydrogenase. McBrien (6) showed that the e.p.r. signal of Cu(II) disappears when Cu(II), as cupric sulfate, is incubated anaerobically with cysteine or yeast alcohol dehydrogenase. Under aerobic conditions the signal stays at its inital intensity. He emphasized that since the incubation with the alcohol dehydrogenase was performed in the absence of substrate or co-factors the reduction of Cu(II) must proceed non-enzymatically, i.e., the protein is serving as a source of non-metabolic reducing equivalents. The capacity of alcohol dehydrogenase to reduce the Cu(II) e.p.r. signal anaerobically was abolished when the sulfhydryl reagent, N-ethylmaleimide, was added to the reaction mixture immediately before the addition of Cu(II). He envisioned

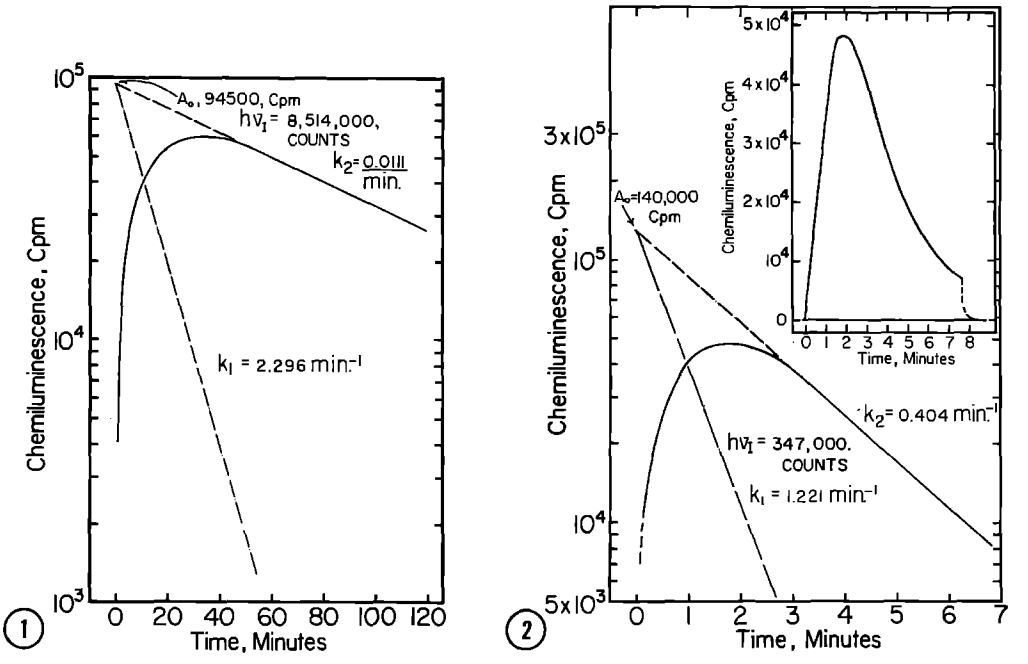


Fig. 1. Semilogarithmic-temporal trace of the copper-dependent chemiluminescence of yeast alcohol dehydrogenase (ADH) evoked by cyanide in the presence of acetaldehyde. System: ADH, 20 μM; PO_4^{--}-buffer, 0.084 M, pH. 7.4; $CuSO_4$, 0.08 mM; CN^-, 0.1 mM; Acetaldehyde, 47.7 mM; final volume, 3 ml.; Temp., 25°C.

Fig. 2. Semilogarithmic and linear (insert) temporal traces of the copper-dependent chemiluminescence fromn spinach ferredoxin evoked by cyanide in the presence of acetaldehyde. System: Ferredoxin, 0.165 mg; $CuSO_4$, 0.084 mM; PO_4^{--}-buffer, 0.084 M, pH 7.4; CN^- = 0.1 mM; Acetaldehyde, 29.6 mM; final volume, 3 ml; Temp., 25°C.

that the reduction of Cu(II) to Cu(I) must proceed by the following reaction:

$$2\ \text{Cu(II)} + 2\ \text{—SH} \longrightarrow 2\ \text{Cu(I)} + \text{-S-S-} + 2\ H^+ \qquad (1)$$

As we describe in the mechanism below, the added cyanide complexes the Cu(I) to initiate the reaction sequence leading to light emission.

Aged, dry alcohol dehydrogenase or old solutions of the enzyme fail to support the light reaction because of the autoxidation of the sulfhydryl groups. When Cu(II) is added to anaerobic solutions of the enzyme the solution becomes cloudy, which does not occur when the Cu(II) is added aerobically.

In Fig. 2 we present the temporal semilog kinetic plots for the light emission from spinach ferredoxin together with an insert of a representative linear, temporal, chemiluminescence response from which the kinetic plot was

made. This compound is highly reducing, E_0' =-0.432V., and is extremely sensitive to traces of oxygen or ferricyanide (4,7,8). The oxidized form will not support chemiluminescence. Since all the iron in native spinach ferredoxin is in the ferric state (8,9,10) it can only be the sulfhydryl moieties furnishing the reducing equivalents to reduce Cu(II) to Cu(I). As a representative non-heme iron-sulfur protein these studies with spinach ferredoxin provide some support for our proposal (11) that it is these loci within the mitochondrial redox chain which are the source of the reducing equivalents facilitating the copper-dependent chemiluminescence evoked by cyanide in the presence of acetaldehyde. It was this work which prompted the studies of this paper.

We present in Fig. 3 the temporal semilog kinetic plot of the chemiluminescene evoked from rabbit liver metallothionein (MT) prepared for Sigma as described by Nordberg et al. (12). This preparation contained 67 µg atoms of Cd and 2.8 µg of Zn per mg protein of the Type I thionein which has a MW of 5608.

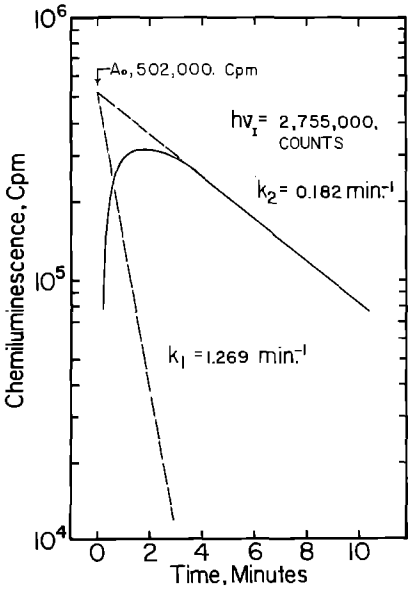
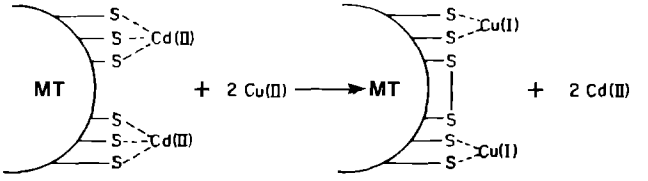
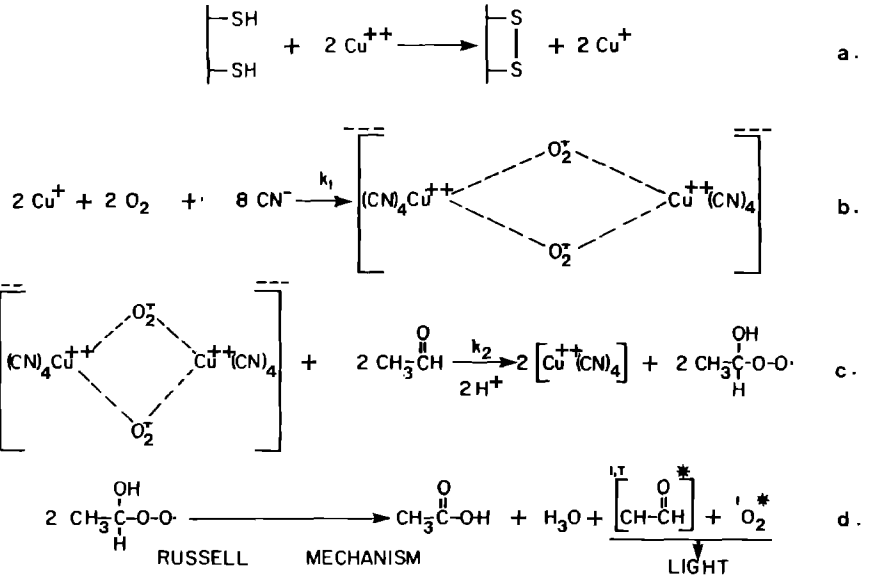


Fig. 3. Semilogarithmic temporal trace of the copper-dependent chemiluminescence of Metallothionein evoked by cyanide in the presence of acetaldehyde. System: metallothionein, 0.165 mg; PO_4^{--}-buffer, 0.084 M, pH 7.4; $CuSO_4$, 0.32 mM; CN^-, 0.1 mM; acetaldehyde, 47.4 mM; final volume, 3 ml; Temp., 25°C.

This preparation failed to chemiluminescene on the addition of acetaldehyde and cyanide. The prior addition of Cu(II) to the system, however, facilitated the acetaldehyde/cyanide evokable emission. Further, we found that the addition of Cu(II) to the preparation resulted in the slow development of the greenish fluorescence at 565 nm, characteristic of the Cu(I)-thiolate complexes (13). It is evident therefore that Cu(II) is being reduced to Cu(I) by free -SH groups or by -SH freed by the displacement of Cd(II) by Cu(II). These redox events probably proceed intramolecularly as proposed by Suzuki and Maitani (14) for the replacement of zinc by copper in the zinc-metallothioneins:



From a knowledge of the redox potentials of several copper-cyanide complexes (15) and a careful kinetic analysis of the copper-facilitated chemiluminescence of mitochondria (11), which appears to proceed by similar reactions, we propose the following mechanism for the copper mediated light emission form the sulfhydryl proteins evoked by cyanide in the presence of acetaldehyde:



In this mechanism the Cu(I), formed by the reduction of Cu(II) by the protein sulfhydryl moieties, is complexed by the added cyanide. This complex then proceeds to reduce O_2 to $O_2^{\cdot}$ which, in turn is intercepted by the added acetaldehyde to form the highly reactive perhydroxyl radical (16). This peroxide then disproportionates exothermically by the Russell Mechanism (17) to yield a high energy electronically excited carbonyl function and/or singlet oxygen which on emissive relaxation to the ground state displays as chemiluminescence.

ACKNOWLEDGMENT

We acknowledge with gratitude that this work was supported by Grant Number 5 R01 AA05714 from the National Institute on Alcohol Abuse and Alcoholism.

REFERENCES

1. Boh, E. E., Baricos, W. H., Bernofsky, C. and Steele, R. H. (1982) J. Bioenergetics Biomembrance 14, 115-133.
2. Boh, E. E. and Steele, R. H. (1981) Fed Proc. 40, p. 1668
3. Steele, R. H. and Sabik, J. (1984) Fed. Proc. 43, p. 1876.
4. Tagawa, K. and Arnon, D. I. (1962) Nature 195, 537-543.
5. Steele, R. H. and Breckinridge, B. S. (1963) Biochem. Biophys. Res. Commun. 10, 396-400.
6. McBrien, D. C. H. (1980) Biological Rules of Copper, Ciba Foundation Symposium 79 (new series), pp. 301-318, Excerpta Media, New York.
7. Petering, D., Fee, J.A. and Palmer, G. (1971) J. Biol. Chem. 246, 643-653.
8. Malkin, R. in Iron Sulfur Proteins (1973) Vol. II, Lovenberg, W., Academic Press, pp. 1-26.
9. Fry, K.T. and Sam Pietro, A. (1963) Natl. Acad. Sci., Natl. Res. Council Pub. 1145, p. 253.
10. Palmer, G. and Sands, R.H. (1966) J. Biol. Chem. 241, 253.
11. Steele, R.H., Sabik, J., Benerito, R.R. and O'Dea, S. (1984) In Press.
12. Nordberg, G.F., Nordberg, M., Piscator, M. and Vesterberg, O. (1972) Biochem. J. 126, 491-498.
13. Anglin, J.H., Jr., Batten, W.H., Raz, A.I. and Sayre, R.M. (1971) Photochem. Photobiol. 13, 279-281.
14. Suzuki, K. T. and Maitani, T. (1981) Biochem. J., 199, 289-295.
15. Milazzo, G., Caroli, S. and Sharma, V.K. (1978) Tables of Standard Electrode Potentials, pp. 22-35, Wiley, New York.
16. Gibian, M.J., Sawyer, D.T., Ungermann, T., Tanapoonholrivat, R. and Morrison, N.M. (1979) J. Am. Chem. Soc. 101, 640-644.
17. Russell, G. A. (1957) J. Am. Chem. Soc. 79, 3871-3877.