Vol. 114, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 29, 1983 Pages 578-583

REDUCTION OF ALLOXAN BY MICROSOMAL ELECTRON TRANSPORT PROTEINS

Ingrid Domke and Willi Weis

Biochemisches Institut der Justus-Liebig-Universität
Friedrichstr. 24, D-6300 Gießen, West Germany

Received April 12, 1983

SUMMARY: Alloxan behaves as a substrate for NADH:ferricytochrome b_5 oxidoreductase (EC 1.6.2.2). The apparent K_m for alloxan was 10 mM in liver microsomes and 20 mM with the enzyme prepared by lysosomal digestion. The apparent K_m for NADH was the same with microsomes and the isolated enzyme (30 µM). The maximum turnover rate was calculated as 426 moles electrons/min x mole enzyme. Cytochrome b_5 was shown to reduce alloxan nonenzymatically.

Alloxan is a mild oxidizing agent (1). During reduction of alloxan, a radical intermediate is formed that disappears by disproportionation leading to alloxan and dialuric acid (2).

The chemical structure of alloxan is similar to that of dehydroascorbic acid. The radical monodehydroascorbate is formed during the reversible redox reaction between dehydroascorbate and ascorbate (3). Like the alloxan radical it disappears by disproportionation (4,5). The proteins participating in the reduction of monodehydroascorbate in liver microsomes are NADH:ferricytochrome b_5 oxidoreductase (EC 1.6.2.2), ascorbate: ferricytochrome b_5 oxidoreductase (EC 1.10.2.1), NADH:monodehydroascorbate oxidoreductase (EC 1.6.5.4), and cytochrome b_5 (6). Because of the comproportionating reaction, dehydroascorbate is reduced in the same way.

In view of similarities between alloxan and dehydroascorbic acid, we investigated the reduction of alloxan by microsomal electron transport proteins.

MATERIALS AND METHODS

CHEMICALS: Dialuric acid was prepared according to the method of Tipson (7). NADH (grade II) was obtained from Boehringer Mannheim GmbH (FRG). All other chemicals were of p.a. grade.

0006-291X/83 $1.50

PREPARATION OF MICROSOMES AND MICROSOMAL PROTEINS: Microsomes were prepared from fresh hog liver as described by Weber et al. (8). Cytochrome b_5 and NADH:ferricytochrome b_5 oxidoreductase were isolated from hog liver microsomes with the aid of detergents according to the methods of Spatz and Strittmatter (9,10). The enzyme without the hydrophobic domain was prepared by lysosomal digestion according to Takesue and Omura (11). Enriched ascorbate:ferricytochrome b_5 oxidoreductase was obtained as described by Scherer and Weis (12). NADPH:cytochrome c reductase (EC 1.6.2.4) was prepared by tryptic digestion and purified by affinity chromatography according to the methods of Omura and Takesue (13), and Yasukochi and Masters (14), respectively.

LABORATORY METHODS: Solutions of alloxan in 1 mM HCl were freshly prepared and stored at 0°C. The reaction between NADH and alloxan was followed in 0.1 M phosphate buffer, pH 6.5 by measuring the decrease of A_{366} after mixing the protein with NADH (end concentration 75 µM) and alloxan (end concentration 25 mM). When evaluating enzyme activities (mU/mg protein or mU/3 ml) NADH oxidation in the absence of protein was subtracted. Measurements of cytochrome b_5 contents and cytochrome b_5 oxidation by alloxan were carried out with an Aminco-Chance dual-wavelength spectrophotometer (American Instruments Co., Silver Spring, Maryland, USA) at 409 and 424 nm. The activities of the enzymes NADH:ferricytochrome b_5 oxidoreductase (measured as NADH:ferricyanide reductase), NADPH:cytochrome c reductase, and NADH:monodehydroascorbate oxidoreductase were measured according to Takesue and Omura (15), Masters et al. (16), and Lumper et al. (17), respectively. All enzymatic measurements were carried out at 25°C. Protein content was determined according to the method of Lowry et al. (18).

RESULTS AND DISCUSSION

Incubation of microsomes or lysosomal-extracted NADH:ferricytochrome b_5 oxidoreductase with alloxan and NADH results in a rapid oxidation of NADH (Table 1). A mixture of only alloxan and NADH gives some direct oxidation of the substance (19). The participation of protein thiols in the enzyme-catalyzed redox reaction is shown by incubation with p-chloromercuribenzoate (Table 1). After heat denaturation of the tested proteins, no enzymatic NADH oxidation by alloxan was observed. When alloxan (25 mM) and NADH (75 µM) were kept constant and the protein concentration was varied, the reaction rate was proportional to the concentration of microsomes or NADH: ferricytochrome b_5 oxidoreductase prepared by lysosomal digestion.

Apparent K_m values for alloxan and NADH and V values were calculated from Lineweaver-Burk plots in the presence of microsomes or lysosomal-extracted NADH:ferricytochrome b_5 oxidoreductase (Fig. 1 and 2). Variation of the concentration of alloxan gave an apparent

Table 1. NADH oxidation by alloxan in the presence of microsomes or isolated electron transfer proteins.

Sample	Enzymatic oxidation of NADH [mU/3 ml]
microsomes	206.6 ± 21.1
microsomes, 1 mM p-chloromercuribenzoate	0
NADH:ferricytochrome b_5 oxidoreductase	64.4 ± 3.3
NADH:ferricytochrome b_5 oxidoreductase, 1 mM p-chloromercuribenzoate	0
NADH:ferricytochrome b_5 oxidoreductase, cytochrome b_5	68.0 ± 6.6
cytochrome b_5	0
NADPH:cytochrome c reductase	0
ascorbate:ferricytochrome b_5 oxidoreductase	0

The assays of 3 ml 0.1 M phosphate buffer, pH 6.5 contained 1 mg microsomal protein or corresponding activities or contents, respectively, of lysosomal-extracted NADH:ferricytochrome b_5 oxidoreductase (7.2 U), NADPH:cytochrome c reductase (140.4 mU), and cytochrome b_5 (0.308 nmol). 0.1 ml of enriched ascorbate:ferricytochrome b_5 oxidoreductase was tested. 1 min. after addition of NADH (end concentration 75 µM), the reaction was started with alloxan (end concentration 25 mM). The results are expressed as mean ± s.d. of 4 different measurements.

K_m of 10 mM with microsomes and 20 mM with the isolated reductase. The apparent K_m for NADH was the same with microsomes and the enzyme (30 µM). The reaction is inhibited by NADH concentrations higher than 100 µM (Fig. 2). The maximum turnover number of the reductase was calculated as 426 moles electrons/min x mole enzyme.

Maximum velocities of NADH oxidation by alloxan differ with microsomes (246,2 mU/3 ml) and the isolated enzyme (77,7 mU/3 ml). Therefore we tested a participation of other microsomal electron transport proteins in the reaction catalyzed by microsomes (Table 1). Cytochrome b_5 did not increase the reaction rate in the presence of NADH:ferricytochrome b_5 oxidoreductase. Alloxan did not behave as a substrate for purified NADPH:cytochrome c reductase or ascorbate:ferricytochrome b_5 oxidoreductase. We prepared the native form of NADH:ferricytochrome b_5 oxidoreductase with the aid of detergents. During the preparation, enrichment of NADH oxidation by alloxan was

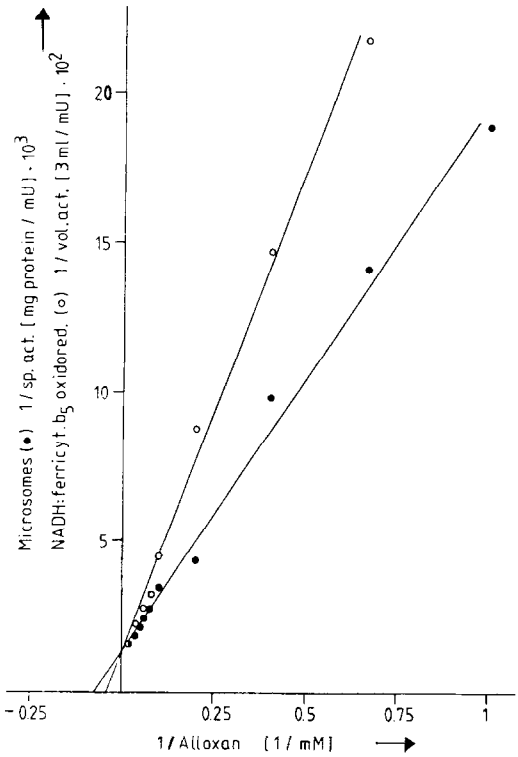

Fig. 1. Lineweaver-Burk plot for calculation of apparent K_m for alloxan in the presence of microsomes or lysosomal-extracted NADH:ferricytochrome b_5 oxidoreductase. The assays contained in 3 ml 0.1 M phosphate buffer, pH 6.5, 1 mg microsomal protein (•) or 8.8 U reductase (o). 1 min. after addition of NADH (end concentration 75 µM), the reaction was started with alloxan. With microsomes a second blank (NADH oxidation in the absence of alloxan) had to be subtracted. The results are expressed as the mean of 5 different measurements.

similar to that of NADH:ferricytochrome b_5 oxidoreductase (measured as NADH:ferricyanide reductase). However the activity of NADH:monodehydroascorbate oxidoreductase did not increase (data not shown). The results suggest that the reaction studied in microsomes is cata lyzed exclusively by NADH:ferricytochrome b_5 oxidoreductase.

Although cytochrome b_5 does not increase enzymatic and nonenzymatic NADH oxidation by alloxan (Table 1), alloxan causes a rapid and complete oxidation of the hemoprotein after exhaustion of reducing equivalents. For testing a possible participation of NADH: ferricytochrome b_5 oxidoreductase in this reaction, cytochrome b_5 was reduced enzymatically with NADH and nonenzymatically with so-

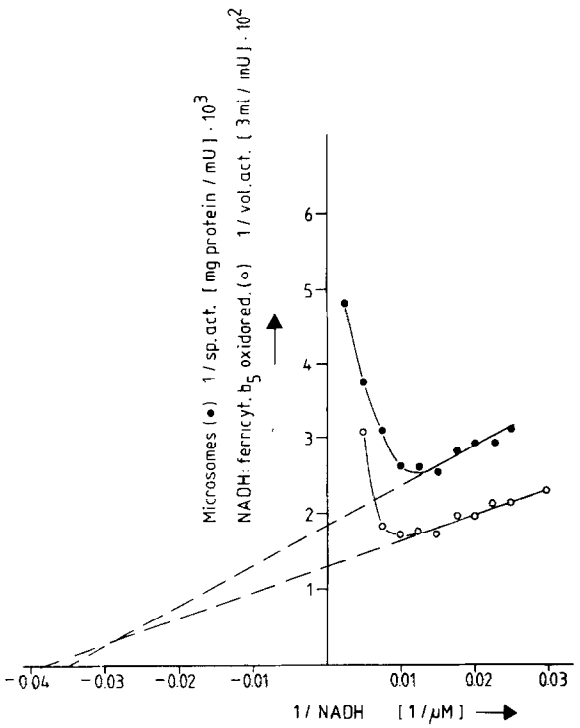

Fig. 2. Lineweaver-Burk plot for calculation of apparent K_m for NADH in the presence of microsomes or lysosomal-extracted NADH:ferricytochrome b_5 oxidoreductase. The assays contained in 3 ml 0.1 phosphate buffer, pH 6.5, 1 mg microsomal protein (•) or 8.8 U reductase (o). 1 min. after addition of NADH, the reaction was started with alloxan (end concentration 25 mM). The results are expressed as the mean of 4 different measurements.

dium dithionite. After addition of alloxan, the velocity of cytochrome b_5 oxidation was the same in the presence or absence of the reductase or the reductase inhibited by p-chloromercuribenzoate (data not shown). These results show that alloxan oxidizes cytochrome b_5 nonenzymatically.

Cytochrome b_5 oxidation by alloxan is biphasic in the presence of NADH and NADH:ferricytochrome b_5 oxidoreductase. The steady state reduction level of the hemoprotein depends on the alloxan concentration (nonenzymatic cytochrome b_5 oxidation) and the activity of the reductase (enzymatic cytochrome b_5 reduction with NADH). Complete cytochrome b_5 oxidation occurs after exhaustion of reducing equivalents.

The participation of an alloxan radical in NADH- and cytochrome b_5 oxidation by alloxan could not be demonstrated by kinetic measure ments. By means of the murexide reaction, we could detect the presence of unstable dialuric acid. Under the same conditions dialuric acid causes a 15% reduction of the hemoprotein (unpublished results) similar to ascorbic acid (6,20).

REFERENCES

1. Richardson,G.M., and Cannan,R.K. (1929) Biochem.J. 23, 68-77.
2. Houée-Levin,C., Gardès-Albert,M., Ferradini,C., and Pucheault, J. (1979) Biochem.Biophys.Res.Comm. 91, 1196-1200.
3. Yamazaki,I., and Piette,L.H. (1961) Biochim.Biophys.Acta 50, 62-69.
4. Weis,W., Zubrzycki,Z., and Staudinger,Hj. (1968) Hoppe-Seyler's Z.Physiol.Chem. 349, 279-282.
5. v.Foerster,G., Weis,W., and Staudinger,Hj. (1965) Liebigs Ann.Chem. 690, 166-169.
6. Weis,W., (1975) Ann.N.Y.Acad.Sci. 258, 190-200.
7. Tipson,S. (1953) Org.Synthesis 33, 3-7.
8. Weber,W., Weis,W., Schaeg,W., and Staudinger,Hj. (1973) Hoppe-Seyler's Z.Physiol.Chem. 354, 1277-1284.
9. Spatz,L., and Strittmatter,P. (1971) Proc.Natl.Acad.Sci.USA 68, 1042-1046.
10. Spatz,L., and Strittmatter,P. (1973) J.Biol.Chem. 248, 793-799.
11. Takesue,S., and Omura,T. (1970) J.Biochem. (Tokyo) 67, 267-276.
12. Scherer,G., and Weis,W. (1977) Hoppe-Seyler's Z.Physiol. Chem. 358, 1499-1503.
13. Omura,T. and Takesue,S. (1970) J.Biochem. (Tokyo) 67, 249-258.
14. Yasukochi,Y., and Masters,B.S.S. (1976) J.Biol.Chem. 251, 5337-5344.
15. Takesue,S., and Omura,T. (1970) J.Biochem. (Tokyo) 67, 259-266.
16. Masters,B.S.S., Williams,C.H.Jr., and Kamin,H. (1967) Methods Enzymol. 10, 565-573.
17. Lumper,L., Schneider,W., and Staudinger,Hj. (1967) Hoppe-Seyler's Z.Physiol.Chem. 348, 323-328.
18. Lowry,O.H., Rosebrough,N.J., Farr,A.L., and Randall,R.J. (1951) J.Biol.Chem. 193, 265-275.
19. Grankvist,K., Marklund,S., Sehlin,J., and Täljedal,I.B. (1979) Biochem.J. 182, 17-25.
20. Everling,F.B., Weis,W., and Staudinger,Hj. (1969) Hoppe-Seyler's Z.Physiol.Chem. 350, 886-888.