MOLECULAR PHARMACOLOGY, 19:184–186



# Comparative Flavoprotein Catalysis of Anthracycline Antibiotic

## Reductive Cleavage and Oxygen Consumption

SU-SHU PAN, LESLIE PEDERSEN, AND NICHOLAS R. BACHUR

*Laboratory of Clinical Biochemistry, Baltimore Cancer Research Program, Division of Cancer Treatment, National Cancer Institute, Baltimore, Maryland 21201*

Received April 11, 1980; Accepted September 2, 1980

SUMMARY

PAN, S., L. PEDERSEN, AND N. R. BACHUR. Comparative flavoprotein catalysis of anthracycline antibiotics; reductive cleavage and oxygen consumption. *Mol. Pharmacol.* **19**:184–186 (1981).

Several flavoenzymes are compared for their ability to catalyze reductive glycosidic cleavage of anthracycline antibiotics and to promote oxygen consumption in the presence of the antibiotics. NADPH cytochrome P-450 reductase, xanthine oxidase, nitrate reductase, NADH cytochrome *c* reductase, and lipoamide dehydrogenase catalyzed with decreasing specific activity the reductive glycosidic cleavage of daunorubicin and showed enhanced oxygen consumption in the presence of daunorubicin. Gluthione reductases, L-amino acid oxidase, D-amino acid oxidase, D-glucose oxidase, and lactic dehydrogenase were inactive. All of those flavoenzymes which catalyze reductive glycosidic cleavage possess single-electron transfer systems.

Reductive glycosidic cleavage of the anthracycline antibiotics adriamycin, daunorubicin, and aclacinomycin A is a major metabolic pathway for these drugs in mammalian systems (1–4) (Fig. 1), resulting in splitting off of the sugar moiety at $^{7}C$ position and the production of deoxyaglycone products (4, 5). We have shown that the flavoenzymes, both microsomal NADPH cytochrome P-450 reductase (6) and milk xanthine oxidase (7), catalyze the reductive glycosidic cleavage reaction, augment oxygen consumption, and catalyze the formation of free radical intermediates of the anthracycline antibiotics. Aerobically, the anthracycline antibiotics serve as an electron shuttle between the flavoenzyme and oxygen, thereby enhancing oxygen consumption (6, 7). Anaerobically, the free radical semiquinones are proposed as intermediates for reductive glycosidic cleavage (8,9). It is known that the reduction of quinones by flavoenzymes may occur either through a one-electron transfer or a two-electron transfer. However, our data from NADPH cytochrome P-450 reductase and xanthine oxidase favor the one-electron transfer mechanism for the reduction of anthraquinones and subsequent oxygen consumption or reductive glycosidic cleavage. Therefore, we wished to determine (a) whether two-electron-transferring flavoenzymes can catalyze reductive glycosidic cleavage and enhance oxygen consumption and (b) whether other one-electron-transferring flavoenzymes are catalytic in the same system. We compared several one- and two-electron-transferring flavoenzymes for their abilities to catalyze reductive glycosidic cleavage and to enhance oxygen consumption with anthracycline antbiotics.

Sources of the anthracycline antibiotics and chemicals have been cited (6, 7). The following enzymes were obtained from Sigma Chemical Company, St. Louis, Mo.: pig heart lipoamide dehydrogenase (NADH: lipoamide oxidoreductase, EC 1.6.4.3); snake venom L-amino acid oxidase (L-amino acid: $O_2$ oxidoreductase, EC 1.4.3.2); D-amino acid oxidase (D-amino acid: $O_2$ oxidoreductase, EC 1.4.3.3); cytochrome *c* reductase (NADH dehydrogenase, EC 1.6.99.3); glutathione reductase (NADPH: glutathione oxidoreductase, EC 1.6.4.2); alcohol dehydrogenase (EC 1.1.1.71); glutamic dehydrogenase (EC 1.1.1.49); and peroxidase (EC 1.11.1.7). *Neurospora* nitrate reductase (NADPH: nitrate oxidoreductase, EC 1.6.6.3) was purified according to the method of Pan and Nason (10). NADPH cytochrome P-450 reductase from Sprague-Dawley male rat liver microsomes was isolated by the method of Yasukochi and Masters (11). Rat liver aldehyde reductase was obtained from Dr. R. Felsted, Baltimore Cancer Research Program.

Enzymatic activities, including oxygen consumption and reductive glycosidic cleavage of anthracycline antibiotics, were measured as previously described (7). The enzymatic reactions were conducted in 0.1 M buffers within the pH range that is considered optimal for their normal enzymatic activities. Among the flavoenzymes

0026-895X/81/010184-03$02.00/0

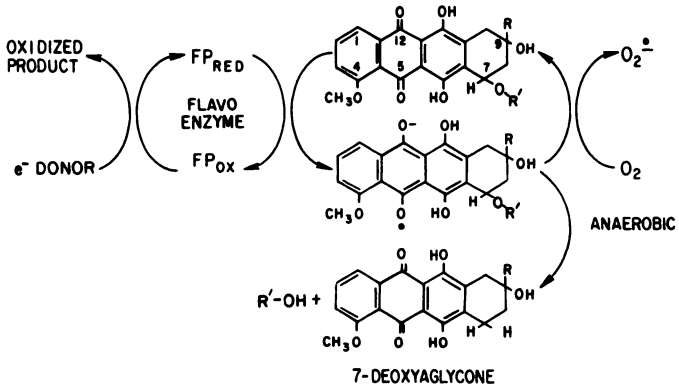


FIG. 1. *Relationship for flavoenyme catalysis of oxygen consumption and reductive glycosidic cleavage*

$FP_{red.}$ flavoprotein reduced; $FP_{ox}$, flavoprotein oxidized; *R*, various substitution groups; *R'*, daunosamine or other sugar moieties.

tested, xanthine oxidase, NADPH-cytochrome P-450 reductase, and NADPH-nitrate reductase were purified to homogeneity. Other enzymes were used as purchased in partially purified form.

As we reported previously, both NADPH cytochrome P-450 reductase and xanthine oxidase catalyze anthracycline reductive cleavage and show augmented oxygen consumption when reduced pyridine nucleotides are cofactors (Table 1). Two additional flavoenzymes, nitrate reductase from *Neurospora* and NADH cytochrome *c* reductase from pig heart, catalyzed reductive glycosidic cleavage of daunorubicin anaerobically to yield deoxydaunorubicin aglycone. Both of these flavoenzymes also catalyzed augmented oxygen consumption with anthracycline antibiotics (Table 1). Both enzymes were unable to transfer electrons from their electron donors to oxygen in the absence of anthracyclines, but oxygen consumption was readily detectable after either daunorubicin or adriamycin was added. A scheme is presented to depict the relationship of oxygen consumption and reductive glycosidic cleavage catalyzed by flavoenzymes (Fig. 1). In contrast, anthracycline antibiotics reduced the oxygen uptake of the other flavoenzymes, L-amino acid oxidase, D-amino acid oxidase, D-glucose oxidase, and lipoamide dehydrogenase (Table 1). Glutathione reductases and lactic dehydrogenase showed minimal or no oxygen uptake. When reductive glycosidic cleavage activities were compared, no cleavage activities were detected for the three oxidases, lactic dehydrogenase, or glutathione reductases. Lipoamide dehydrogenase exhibited a trace of reductive glycosidic activity. Other oxidative-reductive enzymes such as nonflavoprotein rat liver aldehyde reductase (EC 1.1.1.2), alcohol dehydrogenase (EC 1.1.1.71), glutamic dehydrogenase (EC 1.1.1.49), and hemeenzyme peroxidase (EC 1.11.1.7) (not shown) were inactive in both assays.

Reductive glycosidic cleavage activity was correlated to augmentation of oxygen consumption among the flavoenzymes tested. The only exceptions to this were xanthine oxidase (when xanthine was the electron donor) and lipoamide dehydrogenase.

All of our data are for daunorubicin as anthracycline antiobiotic substrate; however, adriamycin functions similarly. Even the product of the glycosidic reductive cleavage reaction, deoxydaunorubicin aglycone, which has the anthraquinone nucleus, functions with the flavoenzymes

TABLE 1

*Flavoenzyme-catalyzed reductive glycosidic cleavage of daunorubicin and oxygen consumption*

Values of enzymatic activities are the average of at least three determinations. Reaction mixtures for both reactions contained electron listed at 1 mM, daunorubicin 0.5 mM, phosphate buffer 0.1 M for all reactions except D-amino acid oxidase. In this case, 0.1 M Tris-HCl was used. Enzyme concentrations varied from one enzyme to another depending on activity.

| Enzyme | Electron donor (1 mM) | pH | Reductive glycosidic cleavage | $O_2$ consumption | |
|---|---|---|---|---|---|
| | | | | Endogenous | Daunorubicin-augmented |
| | | | *μmoles/mg protein/10 min* | *μmoles/mg protein/10 min* | |
| I. Single-electron transport and mixed | | | | | |
| NADPH cytochrome P-450 reductase (rat liver microsome, EC 1.6.2.3) | NADPH | 7.4 | 71.20 | 42.86 | 238.00 |
| Xanthine oxidase (bovine milk, EC 1.2.3.2) | Xanthine | 7.5 | 1.10 | 16.00 | 14.10 |
| | NADH | 7.5 | 2.50 | 0.90 | 3.10 |
| Nitrate reductase (*Neurospora*, EC 1.6.6.3) | NADPH | 7.3 | 0.94 | | |
| | NADH | 7.3 | 0.07 | 0 | 20.00 |
| NADH cytochrome *c* reductase (porcine heart, EC 1.6.99.3) | NADH | 7.5 | 0.97 | 0 | 1.32 |
| II. Two-electron transport | | | | | |
| L-amino acid oxidase (snake venom, EC 1.4.3.2) | L-Leucine | 7.5 | 0 | 9.30 | 7.00 |
| D-amino acid oxidase (hog kidney, EC 1.4.3.3) | D-Leucine | 8.3 | 0 | 32.64 | 20.05 |
| D-glucose oxidase (*Aspergillus*, EC 1.1.3.4) | D-glucose | 6.8 | 0 | 8.00 | 4.40 |
| III. Predominantly two-electron transport | | | | | |
| Lipoamide dehydrogenase (pig heart, EC 1.6.4.3) | NADH | 7.5 | 0.08 | 5.20 | 2.00 |
| Glutathione reductase (wheat germ, EC 1.6.4.2) | NADH | 7.6 | 0 | 0 | 0 |
| Glutathione reductase (yeast, EC 1.6.4.2) | NADH | 7.6 | 0 | 0.08 | 0.11 |
| Lactic dehydrogenase (EC 1.1.1.27) | NADH | 7.3 | 0 | 0 | 0 |

TABLE 2

*Oxygen consumption of xanthine oxidase with NADH as electron donor*

The reactions were carried out as described in Table 1.

| Effector | $O_2$ consumption | |
|---|---|---|
| | Endogenous | Augmented |
| | *μmoles/10 min/mg protein* | |
| Daunorubicin | 0.9 | 3.1 |
| Daunorubicin aglycone | 0.9 | 4.2 |
| Deoxydaunorubicin aglycone | 0.9 | 1.6 |
| Daunosamine | 0.9 | 0.9 |

as an electron carrier to enhance oxygen consumption (Table 2). Daunorubicin aglycone, which has a hydroxyl moiety at $^{7}C$, is a more effective electron carrier than the 7-deoxydaunorubicin aglycone. Daunosamine, the sugar moiety produced from the glycosidic reductive cleavage reaction, was inactive.

When anthracycline is chemically reduced with sodium borohydride, the red color (light absorption at 470 nm) disappears, but reappears upon autooxidation. The colorless state is a property of the hydroquinone or two-electron reduced form. We designed an experiment to determine whether the anthracycline nucleus is reduced to the semiquinone or the hydroquinone form during the enzymatic reduction.

Daunorubicin or daunorubicin aglycone ($1 \times 10^{-5}$ M) was incubated with excess NADPH (1 mM) and NADPH cytochrome P-450 reductase (0.5 μg) under anaerobic conditions; the low concentration of substrates results in concentration of the 7-deoxyaglycone, which remains soluble.The daunorubicin was completely converted to deoxydaunorubicin aglycone rapidly in this reaction, as was the daunorubicin aglycone, and was followed by spectrophotometery. During these reactions, there was no change in the 470nm absorption even up to 48 hr. If hydroquinone were the product, there would be a decrease in 470-nm absorption, as was seen in the chemical reduction. Therefore, we conclude that NADPH cytochrome P-450 reductase catalyzes only the single-electron reduction of the anthracycline and that the hydroquinone form is not produced under these conditions.

When the flavoenzymes are classified according to their electron transfer mechanisms (type I being single and mixed electron transfer and type II being two-electron transfer), a correlation appears between types of electron transfer and reductive glycosidic cleavage activity as well as augmentation of oxygen uptake. The type I enzymes conduct reduction via a semiquinoid enzyme intermediate by a single-electron transfer mechanism (12–14). All four type I flavoenzymes possess reductive glycosidic cleavage activity. The type II flavoproteins, L-amino acid oxidase, D-amino acid oxidase, and D-glucose oxidase, catalyze reductions via a two-electron reduced enzyme (12, 13, 15). They do not catalyze the reductive glycosidic cleavage. Type III flavoproteins, which are predominantly two-electron transfer systems (16, 17), apparently have little reductive cleavage activity and do not augment oxygen uptake by anthracycline antibiotics. We conclude that flavin-containing enzymes equipped with a single-electron transfer system are catalysts for the reductive glycosidic cleavage of anthracycline antibiotics. The flavoproteins, equipped totally or predominantly with a two-electron transfer system, either cannot use anthracycline quinones as their electron acceptors efficiently or they catalyze a two-electron reduction of anthracycline quinones, in which case reductive glycosidic cleavage may not occur.

NADPH cytochrome P-450 reductase, because of its ubiquitous nature in mammalian tissues and its high specific activity for reductive glycosidic cleavage, is the major enzyme responsible for this activity in mammals. However, the wide distribution and significant activities of the type I flavoproteins in cells increase their importance for the biotransformation of these drugs and for free radical production.

ACKNOWLEDGMENT

We wish to thank Ms. Helen Chlewicki in the preparation of the manuscript.

REFERENCES

1. Asbell, M. A., E. Schwartzbach, F. J. Bullock, and D. W. Yesair. Daunomycin and adriamycin metabolism via reductive glycosidic cleavage. *J. Pharmacol. Exp. Ther.* **182**:63–69 (1972).
2. Bachur, N. R., S. L. Gordon, and M. V. Gee. Anthracycline antibiotic augmentation of microsomal electron transport and free radical formation. *Mol. Pharmacol.* **13**:901–910 (1977).
3. Bachur, N. R., and M. V. Gee. Microsomal reductive glycosidase. *J. Pharmacol. Exp. Ther.* **197**:681–686 (1976).
4. Oki, T., T. Komiyama, H. Tone, and T. Inui. Reductive cleavage of anthracycline glycosides by microsomal NADPH cytochrome C reductase. *J. Antibiotics* **30**:613–615 (1977).
5. Bullock F. J., R. J. Bruni, and M. A. Asbell. Identification of new metabolites of daunorubicin and adriamycin. *J. Pharmacol. Exp. Ther.* **182**:70–76 (1972).
6. Bachur, N. R., J. L. Gordon, M. V. Gee, and H. Kon. NADPH cytochrome P-450 reductase activation of quinone anticancer agents to free radicals. *Proc. Natl. Acad. Sci. U. S. A.* **76**:954–957 (1979).
7. Pan, S., and N. R. Bachur. Xanthine oxidase catalyzed reductive cleavage of anthracycline antibiotics and free radical formation. *Mol. Pharmacol.* **17**:95–99 (1980).
8. Bachur, N. R. Anthracycline antibiotic pharmacology and metabolism. *Cancer Treat. Rep.* **63**:817–820 (1979).
9. Mason, R. P. Free radical metabolites of foreign compounds and their toxicological significance, in *Review of Biochemical Toxicology* (Hodgson, Bend, Philpot, eds.). Elsevier, New York, 151–200 (1979).
10. Pan, S., and A. Nason. Purification and characterization of homogeneous assimilatory NADPH-nitrate reductase from *Neurospora crassa*. *Biochim. Biophys. Acta* **523**:297–313 (1978).
11. Yasukochi, Y., and B. S. S. Masters. Some properties of a detergent-solubilized NADPH-cytochrome *c* (cytochrome P-450) reductase purified by biospecific affinity chromatography. *J. Biol. Chem.* **251**:5337–5344 (1976).
12. Yamazaki, I. One-electron and two-electron transfer mechanisms in enzymatic oxidation-reduction reactions. *Adv. Biophys.* **2**:33–76 (1971).
13. Yamazaki, I. Free radicals in enzyme-substrate reactions, in *Free Radicals in Biology* (W. A. Pryor, ed.), Academic Press, Vol. 3. 183–218 (1977).
14. Nakamura, M., and I. Yamakai. One-electron transfer reactions in biological systems. VII. Two types of electron outlets in milk xanthine oxidase. *Biochim. Biophys. Acta* **327**:247–256 (1973).
15. Yagi, K. Red and blue semiquinone of D-amino acid oxidase, in *Reactivity of Flavins* (K. Yagi, ed.), University of Tokyo Press, Tokyo, 29–35 (1975).
16. Williams, C. H. Jr., L. D. Arscott, and E. T. Jones. Active center sequence homology and mechanistic homology between glutathione dehydrogenase, in *Flavin and Flavoproteins* (T. P. Singer, ed.). Elsevier, New York, 455–463 (1976).
17. Nakamura, M., and I. Yamazaki. One-electron transfer reactions in biochemical systems. VI. Changes in electron transfer mechanism of lipoamide dehydrogenase by modification of sulfhydryl groups. *Biochim. Biophys. Acta* **267**:249–257 (1972).

**Send reprint requests to:** Dr. Nicholas R. Bachur, Laboratory of Clinical Biochemistry, Baltimore Cancer Research Program, Division of Cancer Treatment, National Cancer Institute, 655 West Baltimore Street, Baltimore, Md. 21201.