Vol. 167, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1990 Pages 367-372

TRANSPORT OF GLUTATHIONE ACROSS THE MITOCHONDRIAL MEMBRANES

Kazuhei Kurosawa, Norio Hayashi*, Nobuhiro Sato*, Takenobu Kamada*, and Kunio Tagawa[1]

Department of Physiological Chemistry and *First Department of Medicine, Osaka University Medical School, Osaka, Japan

Received January 26, 1990

SUMMARY: Transport of glutathione(GSH) into mitochondria was observed when mitochondria in state 4 respiration were incubated with high concentrations of GSH. This transport was suppressed by antimycin A or dicyclohexylcarbodiimide, or in state 3 respiration. Upon dissipation of the proton gradient by a proton ionophore, mitochondrial GSH was released into the medium. GSH moved freely across the proton-permeated mitochondrial membrane, its movement depending only on the GSH gradient across the inner membrane. These results indicate that there is a transport system for GSH in the mitochondrial membrane, and that a proton gradient is necessary to maintain GSH in the matrix, and to transport GSH into mitochondria.

Glutathione (GSH) is a widely distributed thiol-containing tripeptide that is present in high concentrations, in both the mitochondrial and cytosolic compartments of living cells(1). The existence of a mitochondrial pool of GSH was confirmed by earlier investigators(2, 3), but its origin has been controversial. It is generally accepted that the GSH content of isolated mitochondria is maintained during their preparation or incubation(3, 4). This apparent impermeability of the mitochondrial membrane, and the difference in the half-lives or consumption rates of cytosolic and mitochondrial GSH have led to some groups to conclude that the GSH in the two compartments is completely separated, and to propose the

[1]Correspondence to: Professor Kunio Tagawa, Department of Physiological Chemistry, Osaka University Medical School, 4-3-57, Nakanoshima, Kita-ku, Osaka, 530, Japan.

Abbreviations: GSH, reduced form of glutathione; GSSG, oxidized form of glutathione; CCCP, carbonylcyanide m-chlorophenylhydrazone; Hepes, N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid; DCCD, N,N'-dicyclohexylcarbodiimide.

0006-291X/90 $1.50

idea of mitochondrial GSH-synthesizing systems(4, 5). This idea has, however, been refuted by the demonstration that mitochondria do not contain enzyme activities for GSH synthesis(6).

In this communication, we show that transport of GSH into mitochondria actually occurs when mitochondria in state 4 respiration are incubated with high concentrations of GSH, and that a proton gradient is necessary to maintain GSH in the mitochondrial matrix. Proton movement across the inner membrane seems to have a great influence on the transport of GSH.

MATERIALS AND METHODS

Materials Hepes and CCCP were purchased from Sigma Chemical Co., U.S.A. Sephadex G-50 was from Pharmacia, Sweden. All other chemicals were of analytical grade and were obtained from local sources.
Preparation and incubation of mitochondria Male Sprague-Dawley rats weighing about 250 g were starved overnight, and anesthetized by intraperitoneal injection of sodium pentobarbital at 50 mg/kg body weight. Mitochondria were isolated from their liver by a reported procedure(7), and suspended in 0.25 M sucrose containing 10 mM Hepes-K, pH 7.4. Unless otherwise stated, mitochondria were incubated at 5 mg protein/ml in medium composed of 0.25 M sucrose, 5 mM K-Pi, 10 mM K-succinate, and 10 mM Hepes-K, pH 7.4. In experiments on GSH transport, the potassium salt of GSH was added to the medium, and then at intervals, mitochondria were separated from the medium by a centrifuge column method(8). Briefly, a Sephadex G-50 column (in a disposable tuberculin syringe, 0.6 x 7 cm, equilibrated with incubation medium) was centrifuged before use. The mitochondrial suspension was charged on the column and the column was again centrifuged. In this way, mitochondria separated from the medium were recovered in the effluent. Intramitochondrial GSH was extracted by adding a final concentration of 0.5 N PCA neutralized with KOH. In some experiments mitochondria were removed by applying the incubation mixture to a membrane filter of 0.44 μm pore size (Millex EX-GS, Nihon Millipore Co.Ltd., Japan), and the filtrate was used for assay of GSH released from mitochondria.
Assay method GSH was assayed by a HPLC-electrochemical method: samples were loaded on a reverse phase column (Shimpack CLC-ODS, Shimadzu Co. Ltd., Japan), equilibrated with the developing buffer, 100 mM Na-Pi, pH 2.5, and the GSH in the eluate was monitored with an amperometric detector (E-502, Irika Inc., Japan), with an applied voltage of 0.6 volt. Protein was measured by the method of Lowry et al. (9) with bovine serum albumin as a standard.

RESULTS

Status of mitochondrial GSH in state 4 respiration. Jocelyn reported that isolated mitochondria were capable of retaining GSH in their matrix, but that in certain conditions some of the GSH was released into the external medium(3). We first incubated mitochondria and at intervals measured the GSH in the mitochondria and in the medium separately. As seen

in Fig.1, upon incubation of mitochondria in state 4 respiration, about 25 % of the GSH was released into the medium. The GSH value of 1 nmol/mg protein in the medium corresponded to a concentration of 5 μM GSH in our experimental conditions. Assuming that 1 mg of mitochondrial protein is equivalent to 1 μl of matrix volume(10), the amount of GSH of 3 nmol/mg protein in the mitochondria corresponded to a GSH concentration of 3 mM in the matrix space. This means that mitochondria in state 4 respiration retain a several hundred-fold higher GSH concentration in the matrix than that in the external medium. No other mitochondrial substances such as adenine nucleotides and m-aspartate transaminase were detected in the medium, suggesting that GSH in the medium was probably released from intact mitochondria through channels in the inner membrane, rather than being derived from artificially collapsed mitochondria.

Transport of GSH into mitochondria Mitochondria in state 4 respiration were incubated with various concentrations of GSH, and the contents of

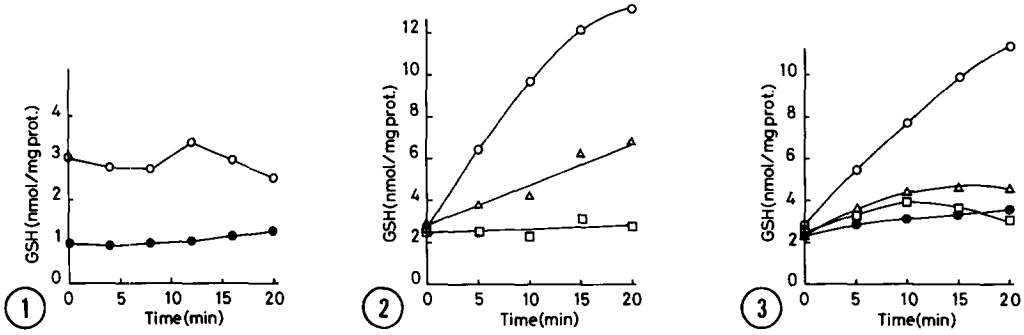

Fig. 1. Status of mitochondrial pool of GSH in state 4 respiration. Mitochondria in state 4 respiration were incubated at 22°C. At the times indicated, they were separated from the medium as described in MATERIALS and METHODS, and GSH in the mitochondria (○) and medium (●) were measured.

Fig. 2. Transport of GSH into mitochondria. The reaction of GSH transport was started by adding GSH at a concentration of 2 mM (□), 5 mM (△), or 10 mM (○) to the medium of mitochondria in state 4 respiration. At the times indicated, mitochondria were separated from the medium as described in MATERIALS and METHODS, and their GSH content was measured.

Fig. 3. Effect of the respiratory state on the transport of GSH into mitochondria. Mitochondria were incubated with 10 mM GSH under various conditions: ○, state 4; △, state 3; □, state 4 + 2 μM antimycin A; ●, state 4 + 50 μM DCCD. State 3 respiration was induced by adding 10 mM ADP to the incubation medium. At the times indicated, mitochondria were separated from the medium as described in MATERIALS and METHODS, and their GSH content was measured.

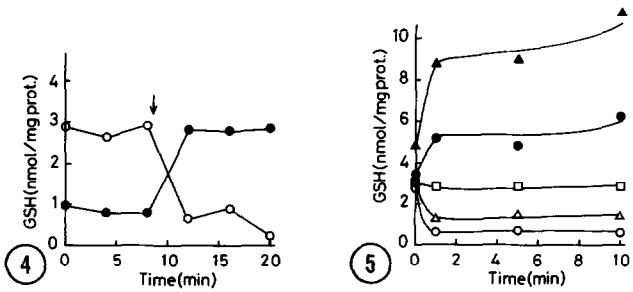

Fig. 4. Effect of CCCP on the status of the mitochondrial pool of GSH. Mitochondria in state 4 respiration were incubated, and the GSH contents of the mitochondria(○) and medium(●) were measured at intervals as described in the legend to Fig.1. CCCP was added at a concentration of 2 μM (indicated by the arrow).

Fig. 5. GSH contents in proton-permeated mitochondria incubated with various concentrations of GSH. Mitochondria were incubated as described in MATERIALS and METHODS, and the reaction of GSH transport was started by simultaneous additions of GSH and 2 μM CCCP. The concentration of the GSH added to the medium was 0 mM (○), 0.5 mM (△), 2 mM (□), 10 mM (●), or 20 mM (▲). At the times indicated, mitochondria were separated from the medium, and their GSH content was measured.

intramitochondrial GSH were measured at intervals. As seen in Fig.2, incubation with high concentrations of GSH resulted in a concentration-dependent transport of GSH into the mitochondria. However, this transport was clearly observed only during state 4 respiration (Fig. 3). It was greatly suppressed in the presence of antimycin A, and during state 3 respiration. Even during state 4 respiration, the presence of DCCD, which probably acted as an ion-channel blocker, suppressed GSH uptake. These results indicate that the transport of GSH into mitochondria is dependent on the proton gradient across the inner membrane.

GSH movement across the membrane of proton-permeated mitochondria When CCCP was added to eliminate the proton gradient, GSH was rapidly released from mitochondria (Fig.4). To clarify the mechanism involved, we incubated proton-permeated mitochondria with various concentrations of GSH, and then measured the GSH content in the mitochondria (Fig.5). In proton-permeated mitochondria, release or uptake of GSH occurred depending on the concentration of GSH in the medium. This finding shows that a proton gradient is necessary to maintain GSH in the matrix space, and that GSH is freely movable across the proton-permeated membrane; its direction of movement is determined only by its own gradient across the inner membrane.

DISCUSSION

The movement of GSH from the outside of mitochondria to the matrix space is actually an unfavorable flow, because GSH is already present at high concentration in the matrix, and a voltage gradient across the inner membrane, with the inside negative and outside positive, is inclined to push GSH out, as GSH is negatively charged at a physiological pH. Thus some motive force is necessary to transport GSH into mitochondria. Our data demonstrate that a high external concentration of GSH and a proton gradient constitute the motive force to bring the external GSH into the mitochondria. As GSH is present at high concentrations in the cytosol of living cells, this motive force presumably functions physiologically.

Several groups have reported evidence for the existence of a mitochondrial anion channel (11, 12, 13). Beavis and Garlid demonstrated the presence of the inner membrane anion channel that had broad specificity for many anions and was controlled by Mg^{2+} and H^{+} in the matrix (12, 14, 15). The GSH transporter described here shares some properties with the anion channel; namely, both are strongly inhibited by DCCD, and GSH can be transported into the respiring mitochondria in the presence of valinomycin and potassium (data not shown), which also accelerate anion transport due to elevation of the pH in the matrix(16). However, in non-respiring mitochondria in which the anion channel had been opened by depletion of Mg^{2+} with A23187(17, 18), valinomycin and potassium did not induce the GSH transport (data not shown), in contrast to other anions(14). Thus, it is unlikely that the two channels are identical, although at present this possibility is not completely ruled out.

Tahiliani recently reported that mitochondrial coenzyme A transport is carried out with the aid of the electrical gradient of the membrane (19). Unlike GSH transport, however, this transport was inhibited by phosphate.

The transport of GSSG is also an interesting subject. Olafsdottir and Reed reported that there is no GSSG channel in the mitochondrial membrane (20). However, we observed that if mitochondria in state 4 respiration

were incubated with GSSG, the GSH levels in the mitochondria and the medium increased. This indicates that GSSG is also incorporated into mitochondria and is reduced to GSH by oxidation of NADPH, catalyzed by GSSG reductase. We are now characterizing the glutathione channel.

ACKNOWLEDGMENT

This study was supported in part by a Grant-in-Aid for Scientific Research (A63440025) from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. Meister,A. (1988) J. Biol. Chem. 263, 17205-17208.
2. Vignais,P.M. and Vignais,P.V. (1973) Biochim. Biophys. Acta 325, 357-374.
3. Jocelyn,P.C. (1975) Biochim. Biophys. Acta 369, 427-436.
4. Wahllander,A., Soboll,S. and Sies,H. (1979) FEBS Lett. 97, 138-140.
5. Meredith,M.J. and Reed,D.J. (1982) J. Biol. Chem. 257, 3747-3753.
6. Griffith,O.W. and Meister,A. (1985) Proc. Natl. Acad. Sci. USA 82, 4668-4672.
7. Johnson,D. and Lardy,H. (1967) Methods Enzymol. 10, 94-96.
8. Penefsky,H.S. (1977) J. Biol. Chem. 252, 2891-2899.
9. Lowry,O.H., Rosebrough,N.J., Farr,A.L. and Randall,R.J. (1951) J. Biol. Chem. 193, 265-275.
10. Beavis,A.D., Brannan,R.D. and Garlid,K.D. (1985) J. Biol. Chem. 260, 13424-13433.
11. Selwyn,M.J., Dawson,A.P. and Fulton,D.V. (1979) Biochem. Soc. Trans. 7, 216-219.
12. Garlid,K.D. and Beavis,A.D. (1986) Biochim. Biophys. Acta 853, 187-204.
13. Sorgato,M.C., Keller,B.U. and Stuhmer,W. (1987) Nature 330, 498-500.
14. Beavis,A.D. and Garlid,K.D. (1987) J. Biol. Chem. 262, 15085-15093.
15. Beavis,A.D. and Powers,M.F. (1989) J. Biol. Chem. 264, 17148-17155.
16. Beavis,A.D. and Garlid,K.D. (1988) J. Biol. Chem. 263, 7574-7580.
17. Dordick,R.S., Brierley,G.P. and Garlid,K.D. (1980) J. Biol. Chem. 255, 10299-10305.
18. Reed,P.W. and Lardy,H.A. (1972) J. Biol. Chem. 247, 6970-6977.
19. Tahiliani,A.G. (1989) J. Biol. Chem. 264,18426-18432.
20. Olafsdottir,K. and Reed,D. (1988) Biochim. Biophys. Acta 964, 377-382.