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SPECTROSCOPIC STUDY OF HYDROXYPROLINE TRANSPORT IN RAT KIDNEY MITOCHONDRIA

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SUMMARY Hydroxyproline uptake by rat kidney mitochondria is here first shown by monitoring the reduction of the intramitochondrial pyridine nucleotides which occurs as a result of metabolism of imported hydroxyproline via hydroxyproline oxidase and 3-hydroxy-pyrroline-5-carboxylate dehydrogenase. Widely used criteria for demonstrating the occurrence of carrier-mediated transport were applied to this process. Hydroxyproline uptake shows saturation features (Km and Vmax values, measured at 20°C and at pH 7.20, were found to be about 1.4 mM and 5 nmoles/min x mg mitochondrial protein, respectively) and proves to be inhibited by the impermeable compound phenylsuccinate, but insensitive to externally added methylglutamate. Difference found in the Km and Vmax values, a different inhibitor sensitivity and the failure of hydroxyproline to cause efflux of glutamate from the mitochondria show that hydroxyproline enters mitochondria by means of a translocator different from those which transport proline.

Given the intramitochondrial localization of many enzymes, metabolite movement across the mitochondrial membrane is essential to both mitochondrial and cytosol metabolism. Although anion translocation across mitochondrial membranes has been widely investigated (1, 2), there is still no exhaustive information on transport in RKM (3). In addition to many translocators (4-11), we have recently investigated aminoacid transport in kidney mitochondria, thus showing the existence of the electrophoretic ornithine/Pi antiporter (12, 13); moreover three carriers for glutamine in acidotic mitochondria (14, 15) have already been proposed and studied

ABBREVIATIONS : CH_3GLU, methylglutamate; e.u. enzymatic units; GDH, glutamate dehydrogenase; GDS, glutamate detecting system; GLU, glutamate; GSA, glutamate semialdehyde; HYP, hydroxyproline; 3-OH-P5C, 3-hydroxy-pyrroline-5-carboxylate; 4-OH-GLU, 4-hydroxy-glutamate; P5C, pyrroline-5-carboxylate; PHESUCC, phenylsuccinate; PRO, proline; RKM, rat kidney mitochondria.

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in details. Among the glutamate-linked aminoacids, proline has recently shown to enter RKM by means of two separate translocators, i.e. the PRO uniporter and the PRO/glutamate antiporter (9).

On the other hand the permeation of HYP in RKM has net yet been investigated. HYP released by gross collagen breakdown and subsequent hydrolysis of HYP-containing peptides cannot be directly reutilized for protein synthesis, thus it is committed to excretion or catabolism (16): in this case transport in mitochondria is strictly required. In fact, HYP is oxidized to 3-OH-P5C by a mitochondrial enzymatic mechanism similar to that mediating PRO oxidation (17). The reaction product can then be converted to 4-OH-glutamate by an NAD-dependent dehydrogenase, which is the only common enzyme catalyzing the dehydrogenation reaction for both PRO and HYP mitochondrial degradation (16, 18).

Thus this paper deals with experiments carried out to investigate the mechanism by which HYP enters RKM. The occurrence of a HYP uniporter, different from the two PRO translocators (9), has been demonstrated.

MATERIALS AND METHODS **Materials:** All the reagents used were from SIGMA (St. Louis, U.S.A.). Mitochondrial substrates were used as Tris salts at pH 7.0-7.4.

Mitochondrial preparation: RKM were isolated from male Wistar rats (150-200 g) according to (19) using a medium consisting of 0.25 M sucrose, 20 mM Tris-HCl pH 7.25 and 1 mM EGTA. The final mitochondrial pellet was suspended in the isolation medium to obtain 30-40 mg protein/ml with mitochondrial protein measured according to (20).

Fluorimetric and photometric assays: Changes in the redox state of the pyridine nucleotide were followed either fluorimetrically, using a Perkin-Elmer luminometer LS-5 with excitation and emission wavelengths set at 334 nm and 456 nm respectively, or photometrically, using a Perkin-Elmer Lambda 5 spectrophotometer. In this case ε_{340} value measured for NADH under our experimental conditions was found to be 6.5 mM^{-1} cm^{-1}. Both HYP and PRO uptake was monitored following intramitochondrial $NAD(P)^+$ reduction caused by externally adding the substrate to mitochondria previously incubated with 1.25 μM FCCP, to increase the intramitochondrial $NAD(P)^+$ concentration, and added, 2 min later, with 2 μg rotenone, to prevent the oxidation of the newly synthesized NAD(P)H via the respiratory chain. Glutamate efflux was monitored essentially according to (9, 21) by adding the glutamate detecting system (GDS), consisting of NAD^+ (1 mM) plus GDH (2 e.u.), and then following NAD^+ reduction caused by externally added substrates. The rates of both fluorescence and absorbance changes were obtained as tangents to the initial part of the experimental curve and expressed as nmol intramitochondrial $NAD(P)^+$ (or extramitochondrial NAD^+) reduced/min x mg mitochondrial protein. In order to obtain a quantitative measurement of the rate of intramitochondrial $NAD(P)^+$ reduction, the fluorimetric response was calibrated as in (22).

RESULTS AND DISCUSSION To investigate HYP uptake and metabolism, experiments were carried out by means of fluorimetric and photometric techniques under conditions in which mitochondrial metabolism is essentially allowed to occur. In the same experiment HYP and PRO were compared with respect to certain uptake features. The addition of either HYP or PRO (1 mM each) to RKM preincubated with the uncoupler FCCP and then added with rotenone causes a rapid increase of fluorescence of the intramitochondrial pyridine nucleotides (Fig. 1, A and B). Interestingly, phenylsuccinate (5 mM), an impermeable inhibitor of certain mitochondrial carriers (10, 11, 23-25), which had no effect on the initial rate of change of fluorescence caused by PRO (Fig. 1B and ref. 9), significantly decreases (80%) the rate of intramitochondrial

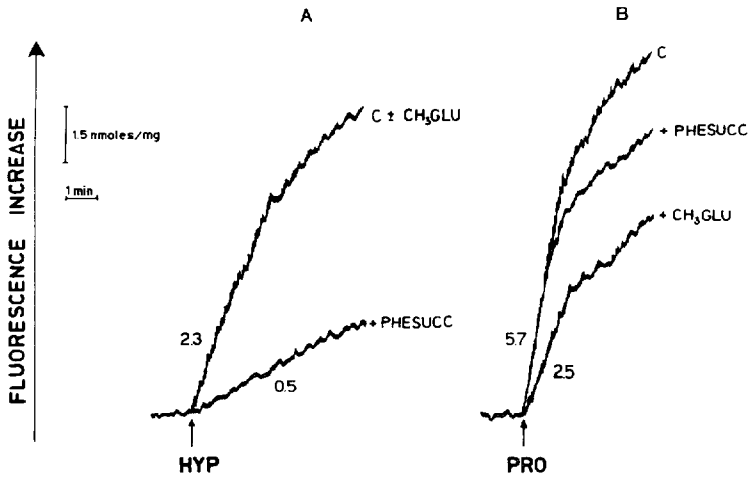


FIGURE 1. Fluorimetric investigation of the HYP uptake by RKM.
RKM (1.5 mg protein) were preincubated at 20°C for 1 min in 2.0 ml of standard medium (consisting of 0.2 M sucrose, 10 mM KCl, 20 mM HEPES-Tris, pH 7.2, 1 mM $MgCl_2$) in the presence of 1.25 μM FCCP and 2 μg rotenone (for details see Methods).
Where indicated, additions were as follows: hydroxyproline (1.5 mM, HYP), proline (1.5 mM, PRO). Phenylsuccinate (5 mM, PHESUCC) and methylglutamate (5 mM, CH_3GLU) were added 1 min before the substrate addition.
The rate, measured as the tangent to the initial part of the progress curve, is expressed as nmoles $NAD(P)^+$ reduced/min x mg mitochondrial protein.

$NAD(P)^+$ reduction due to HYP (Fig. 1A). Contrarily, methylglutamate (5 mM) was found to inhibit the PRO uptake (1B) as expected (9), without affecting HYP uptake. The observed inhibitor sensitivity first suggests that HYP transport is a carrier-mediated process and that the rate of fluorescence increase of RKM added with either HYP or PRO is dependent on two different processes. Thus, a possible explanation of Fig. 1 is as follows (SCHEME 1): HYP enters mitochondria via a carrier (sensitive to phenylsuccinate, but insensitive to methylglutamate); in the matrix HYP is oxidized to 4-OH-glutamate via HYP oxidase and 3-OH-P5C dehydrogenase with reduction of the intramitochondrial $NAD(P)^+$.
Triton experiments [see 9, 12, 21] fail to ascertain whether the rate of HYP oxidation reflects the rate of the transport rather than the rate of one intramitochondrial reaction, since externally added Triton completely prevents any change of fluorescence (not shown). Therefore, in order both to find out whether HYP transport in these experiments can limit HYP oxidation, i.e. to ascertain that the rate of change of fluorescence reflects the rate of HYP uptake, control strength criterion was applied [see 1, 9, 26] by using the inhibitor phenylsuccinate. Phenylsuccinate was found to be a competitive inhibitor of the investigated reaction (Ki = 350 μM) as shown in Fig. 2, where the rate of fluorescence increase was measured as a function of phenylsuccinate concentration in the presence of either 2.5 or 5 mM HYP. It should be noted that if the reciprocal of the rate of investigated reactions is plotted against inhibitor concentration, the resulting Dixon plot extrapolated to zero inhibition provides a measure of transport in the

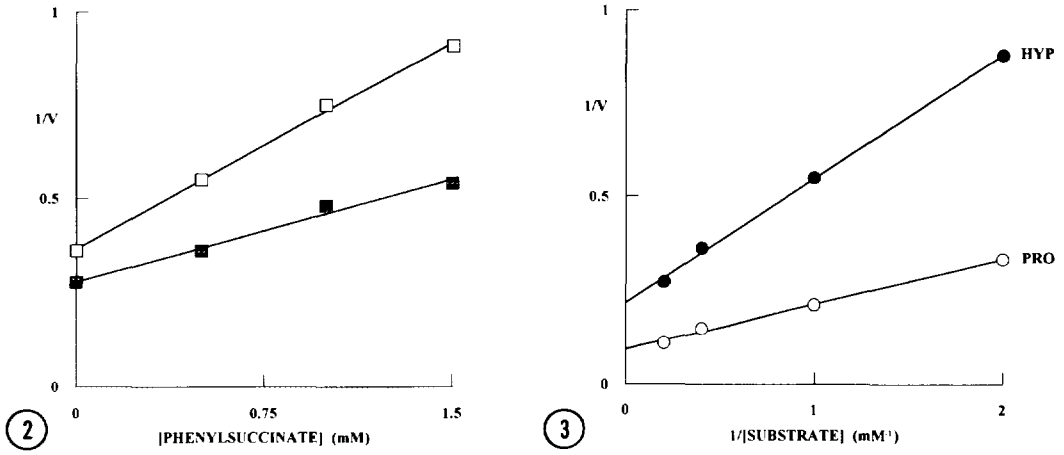
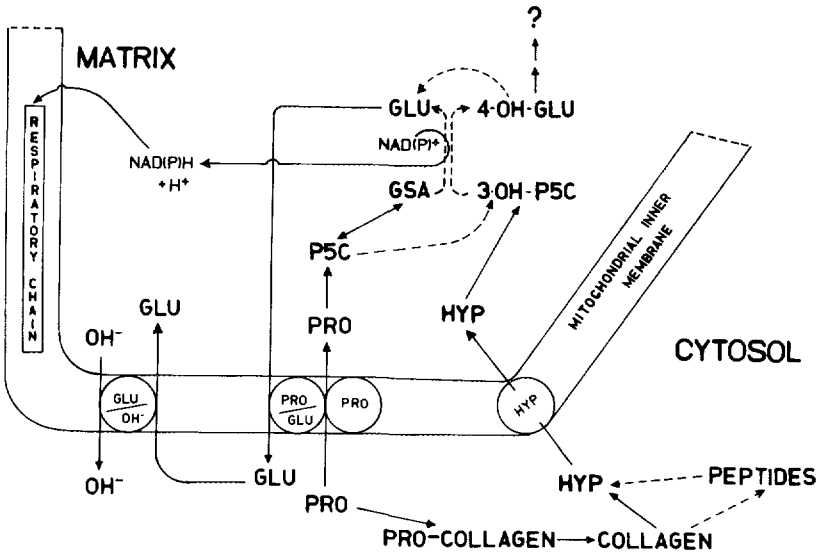


FIGURE 2. Dixon plot of the inhibition by phenylsuccinate of the rate of reduction of intramitochondrial NAD(P)$^+$ due to externally added HYP.
RKM (1.3 mg protein) were preincubated at 20°C for 1 min in 2.0 ml of standard medium in the presence of 1.25 μM FCCP and 2 μg rotenone. The reduction rate of intramitochondrial NAD(P)$^+$ was measured by using 2.5 mM (□) and 5 mM (■) HYP in the absence or presence of phenylsuccinate at the indicated concentration.
The rate is expressed as nmoles NAD(P)$^+$ reduced /min x mg mitochondrial protein.

FIGURE 3. Kinetic analysis of HYP uptake using the double reciprocal plot.
RKM (1.5 mg protein) were preincubated at 20°C for 1 min in 2 ml of standard medium in the presence of 1.25 μM FCCP and 2 μg rotenone. The experiment was carried out as described in Fig. 1. Hydroxyproline (●) and proline (○) were added at the indicated concentrations.
The rate, measured as the tangent to the initial part of the progress curve, is expressed as nmoles NAD(P)$^+$ reduced/min x mg mitochondrial protein.

absence of the inhibitor [see 1, 9, 26]. Statistical analysis shows that the intercept to the ordinate axis of the line given by linear regression analysis of the experimental points obtained in the presence of inhibitor coincides with the experimental point calculated in the absence of phenylsuccinate, thus demonstrating that the measured increase of fluorescence reflects the rate of transport across the mitochondrial membrane.

The dependence of the rate of uptake of either HYP or PRO on increasing substrate concentration was studied by means of the double reciprocal plot (Fig. 3). Both HYP and PRO uptake reveals hyperbolic saturation characteristics. Km and Vmax values for HYP and PRO were found to be 1.4 mM and 5 nmol/min x mitochondrial protein and 1.2 mM and 10 nmol/min x mg protein for HYP and PRO uptake, respectively. Km value for HYP, which is probably within the physiological free HYP concentration range (16, 27, 28), favours the possible occurrence of HYP carrier-mediated translocation *in vivo*; on the other hand the Vmax value is similar to those reported for other kidney carriers (1, 4-15). The observed difference in Vmax, which is a specific feature of each carrier (see 1, 2), confirms that the HYP and PRO uptake do not share the same carriers, i.e. that uptake of HYP occurs via a carrier different from those which can translocate proline (9).



SCHEME 1

To gain further insight into the mechanism of HYP uptake into RKM the capability of HYP to cause efflux of glutamate synthesized in the matrix as a result of HYP uptake and metabolism (see SCHEME 1) was tested (Fig. 4). This was achieved by using already reported methods (9)

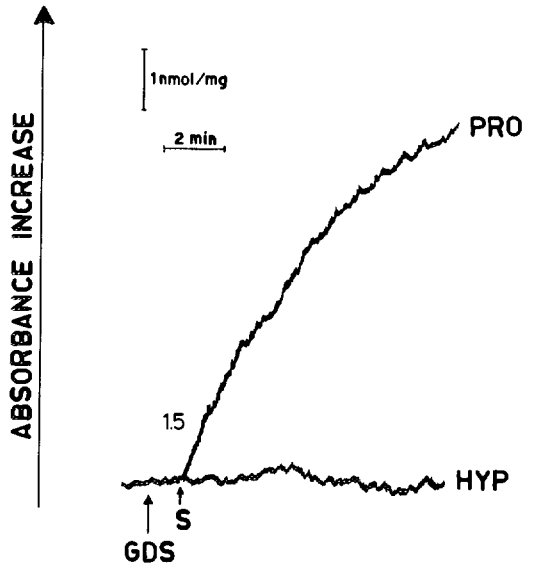


FIGURE 4. Failure of externally added HYP to cause glutamate appearance outside mitochondria.

RKM (1.5 mg protein) were preincubated at 20 °C for 1 min in 2 ml of standard medium in the presence of 1.25 μM FCCP and 2 μg rotenone. Additions, where indicated, were as follows: hydroxyproline (1 mM, HYP), proline (1 mM, PRO), glutamate detecting system (GDS) (consisting of 1 mM NAD^+ plus 2 e.u. glutamate dehydrogenase).

The rate is expressed as nmoles extramitochondrial NAD^+ reduced/min x mg mitochondrial protein.

in which the appearance of glutamate in the extramitochondrial phase was monitored by measuring the increase in the absorbance of NADH in the presence of glutamate dehydrogenase (see Methods). HYP fails to cause the efflux of intramitochondrial glutamate, produced by 4-OH-GLU (see 16), formed in the matrix from the taken-up HYP, as shown by the constancy of absorbance. As a control, PRO/glutamate exchange, whose occurrence has been shown recently (9), was monitored in the same experiment (Fig. 4). As expected, as a result of PRO addition an increase of absorbance is observed, which shows the appearance of glutamate outside mitochondria. These results further confirm that HYP does share neither PRO uniporter (see FIGG. 1 and 3), neither PRO/glutamate antiporter (9) to enter mitochondria.

In another experiment, no efflux of glutamate from ^{14}C-glutamate-loaded RKM was found following HYP addition to mitochondria (not shown).

In conclusion, we demonstrate in this paper that HYP catabolism is accomplished by the specific HYP translocator and by the intramitochondrial enzymes. Whether the intermediates of HYP degradation could affect the metabolic flux of the PRO intermediates (see SCHEME 1 and refs. 16, 29) remains to be established.

REFERENCES

1. LaNoue, K.F., and Schoolwerth, A.C. (1984) in Comprehensive Biochem.: Bioenergetics pp.221-261. Elsevier, Amsterdam, New York, Oxford.
2. Kramer, R., and Palmieri, F. (1992) Molecular mechanisms in Bioenergetics (L.Ernster Ed.) Elsevier Science Publishers pp. 359-384.
3. Schoolwerth, A.C., and LaNoue, K.F. (1985) Annu. Rev. Physiol. 47, 143-171.
4. Schoolwerth, A.C., Nazar, B.L., and LaNoue, K.F. (1978) J. Biol Chem. 253, 6177-6183.
5. Cheema-Dhadli, S., and Halperin, M.L. (1979) Eur. J. Biochem. 99, 483-489.
6. Brosnam, J.T., Redmond, W., Morgan, D., and Whalen, P. (1980) Int. J. Biochem. 12, 139-143.
7. Atlante, A., Passarella, S., Minervini, M., Pierro, P., and Quagliariello, E. (1993) Abstr. XX Congr. Naz. GIBB, Bari, Italy.
8. Schoolwerth, A.C., LaNoue, K.F., and Hoover, W.J. (1983) J. Biol. Chem. 258, 1735-1739.
9. Atlante, A., Passarella, S., Pierro, P., and Quagliariello, E. (1994) Arch. Biochem. Biophys. 309, 139-148.
10. Passarella, S., Simone, M.S., Mancini, C., and Quagliariello, E. (1983) Bull., Mol. Biol. Med. 8, 231-240.
11. Passarella, S., Atlante, A., and Quagliariello, E. (1985) Biochem. Biophys. Res. Commun. 129, 1-10.
12. Passarella, S., Atlante, A., and Quagliariello, E. (1989) Biochem. Biophys. Res. Commun. 158, 870-879.
13. Passarella, S., Atlante, A., and Quagliariello, E. (1990) Eur. J. Biochem. 193, 221-227.
14. Passarella, S., Atlante, A., and Quagliariello, E. (1992) Molecular mechanisms of transport (Quagliariello, E., and Palmieri, F. Eds.) Elsevier Science Publishers. pp. 143-150.
15. Atlante, A., and Passarella, S. (1993) Abstr. Workshop "New Perspectives in Mitochondrial Research", Padova, Italy, p.47.
16. Adams, E., and Frank, L. (1980) Ann. Rev. Biochem. 49, 1005-1061.
17. Adams, E., and Goldstone, A. (1960) J. Biol. Chem. 235, 3499-3503.
18. Valle, D., Goodman, S.I., Harris, S.C., and Phang, J.M. (1979) J. Clin. Invest. 64, 1365-1370.
19. LaNoue, F.K., Nicklas, J., and Williamson, J.R. (1970) J. Biol. Chem. 245, 102-111.
20. Waddel, W.J., and Hill, C. (1956) J. Lab. Clin. Med. 48, 311-314.
21. Passarella, S., Atlante, A., Barile, M, and Quagliariello, E. (1984) Biochem. Biophys. Res. Commun. 121, 770-778.
22. Gimpel, J.A., De Haan, E.J., and Tager, J.M. (1973) Biochim. Biophys. Acta 292, 582-591.

23. Atlante, A., Passarella, S., Giannattasio, S., and Quagliariello, E. (1985) Biochem. Biophys. Res. Commun. 132, 8-18.
24. Passarella, S., Palmieri, F., and Quagliariello, E. (1978) FEBS Lett. 90, 61-64.
25. Passarella, S., Palmieri, F., and Quagliariello, E. (1977) Arch. Biochem. Biophys. 180, 160-168.
26. Passarella, S., Ostuni, A., Atlante, A., and Quagliariello, E. (1988) Biochem. Biophys. Res. Commun. 156, 978-986.
27. Kibrick, A.C., Kitagawa, G., Maskaleris, M.L., Gaines, J.R., and Milhorat, A.T. (1965) Proc. Soc. Exp. Biol. Med. 119, 622-624.
28. Oye, I. (1962) Scand. J. Clin. Lab. Invest 14, 259-262.
29. Kramar, R., and Fitscha, P. (1970) Enzymologia 39, 101-108.