THE ENZYMATIC CONVERSION OF γ-OH-α-KETOGLUTARATE (HKG) TO MALATE: (A postulated step in the cyclic oxidation of glyoxylate)[1]

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The non-enzymatic formation of HKG from glyoxylate and oxalacetate (OAA) has been demonstrated (Payes and Laties, 1963) as has its synthesis from glyoxylate and pyruvate by an enzyme from rat liver (Kuratomi and Fukunaga, 1960), and by extracts of plant tissues (Payes and Laties, unpublished). The enzymatic conversion of HKG to malate by a mitochondrial dehydrogenase and by a peroxidase is described below. HKG has been shown to be an effective competitive inhibitor of several enzymes of the TCA cycle (Payes and Laties, 1963). In view of the inhibitory characteristics of HKG, and the fact that HKG may be synthesized and metabolized by the same tissue, HKG may be considered as a potential regulator of respiratory metabolism. Furthermore, with the demonstrated conversion of HKG to malate there is the interesting possibility that HKG represents an intermediate in the pyruvate-catalyzed cyclic oxidation of glyoxylate.

Materials and Methods

Plant mitochondria were prepared according to Biale et al. (1957) and rat liver mitochondria according to the method of Schneider (1948). Acetone powders of plant and liver mitochondria, yeast and Chlorella were prepared according to Morton (1955). Potato peroxidase was prepared

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essentially by the method used for horse radish peroxidase by Kenten and Mann (1954). In the case of potato peroxidase the polyphenols had first to be removed in order to obtain an active preparation. Polyphenols were removed as follows: Potato tissue was homogenized in a blendor with 0.02M diethyl dithiocarbamate. To the supernatant obtained after centrifugation, Nuchar charcoal processed with stearic acid [Alm (1952); adapted by Dr. W. R. Rees for separation of phenols] was added and stirred for 5 minutes. After centrifugation at high speed a clear supernatant free of phenolics was obtained from which peroxidase was prepared as indicated. Glycolic acid oxidase was obtained from tobacco leaves by the method of Zelitch and Ochoa (1953). The synthesis of HKG and its isolation were as described earlier by Payes and Laties (1963). Glyoxylate and OAA in equal quantities in 0.001 M $MgSO_4$ were brought to pH 7.4 and allowed to react for 1.5 hours. Sufficient cation resin (Amberlite IR 120 in the acid form) was then added to remove all cations, a step which is crucial to the isolation. After water was evaporated at room temperature the reaction product was handled as described earlier.

Synthesis of C^{14}-labeled HKG: 1-C^{14} and 2-C^{14}-glyoxylate, generated by the action of glycolic oxidase on appropriately labeled glycolate, were incubated with unlabeled oxalacetate to yield 5-C^{14} and 4-C^{14} HKG respectively. Incubation was carried out in tris buffer, pH 8.0, in the presence of Mg^{++} and sufficient catalase to degrade the H_2O_2 formed in glycolate oxidation. HKG oxidation was followed by measuring oxygen consumption polarographically, or by measuring ferricyanide reduction spectrophotometrically by noting the increase in O.D. at 625 mμ following the formation of Prussian blue by the addition of 1.0 μmole ferric chloride in a volume brought to 5.0 ml. Without enzymic ferricyanide reduction the O.D. of the control was in each case vanishingly low.

The product of HKG oxidation: At the end of a suitable period the incubation mixture of HKG and enzyme was passed through a cation resin, concentrated and chromatographed, the developing solvent being the organic

phase of a 1:1:1 (by volume) mixture of butanol/formic acid/H_2O aged for 24 hrs. The single oxidation product — well separated from HKG — was identified as malate, eluted, and totally transferred to suitable planchets for the estimation of radioactivity.

Results

As demonstrated in Table I, the oxidation of HKG by potato peroxidase depends upon a catalytic quantity of an appropriate phenol. Labeled malate is the sole product of the oxidation of either C_4 or C_5 labeled HKG by potato peroxidase (Table II).

Table I

Oxidation of HKG by potato peroxidase

	μl O_2/hr (calculated from the initial rate)
— + phenol	0
HKG + —	0
HKG + phenol	108
HKG + p-OH benzaldehyde	58
HKG + phenolic fraction of potato	30

Reaction mixture: 25 μmoles acetate buffer, pH 5.0; 1.8 μmoles $MnCl_2$, 0.1 μmole indicated phenol, HKG 3 μmoles, enzyme from 2 g F.W. tissue. Final volume of reaction mixture, 3 ml.

Table II

Oxidation of labeled HKG by potato peroxidase

	Initial cpm in HKG	cpm in malate
2.5 μmoles 5-C^{14}-HKG + enzyme	28,600	13,000
" " + boiled enzyme	"	0
2.5 μmoles 4-C^{14}-HKG + enzyme	12,000	6,150
" " + boiled enzyme	"	0

Reaction conditions as in Table I; 0.1 μ mole phenol, reaction time 1 hr. Counts in malate after chromatographic isolation (see Materials and Methods).

Besides peroxidase, an NAD-dependent mitochondrial enzyme oxidizes HKG to yield malate as the only demonstrable product. Although the oxidation is analogous to the oxidation of α-ketoglutarate to yield succinate, the enzyme involved is distinguishable from α-ketoglutarate dehydrogenase with respect to resistance to freezing (Table III), relative prevalence (Table V), and pH optimum (Table VI). While once-washed mitochondria give a limited indication of the cofactor requirements for HKG dehydrogenase (Table III), acetone powder extracts show marked cofactor responses typical of an α-keto dehydrogenase (Table IV).

Table III

Comparison of oxidation of HKG and αKG by sweet potato mitochondria

	Mitochondria freshly prepared		Mitochondria after being frozen for 24 hrs.	
Addition Substrate:	α-KG	HKG	α-KG	HKG
	μl O_2/hr			
None	0	0	0	0
NAD	50	35	39	55
NAD + ADP	156	48	88	68
NAD + ADP + TPP	269	57	152	75
NAD + ADP + TPP + CoA	293	65	176	80
As above, substrate omitted	0	0	0	0

Substrate 6 μmoles; NAD, ADP 1 μmole, TPP 0.01 μmole, Mg^{++} 3 μmoles, K-phosphate, pH 7.2, 60 μmoles; sucrose 900 μmoles; mitochondria equivalent to 0.4 mg protein N; total volume 3.0 ml. Oxygen uptake measured polarographically.

HKG oxidation does not require NAD when ferricyanide serves as electron acceptor, while malate oxidation does. Consequently when NAD is withheld, malate accumulates as the product of HKG oxidation, and can be shown to be labeled when C_4 or C_5 labeled HKG is the substrate (Table IV). The bulk of the radioactivity initially provided is to be found in residual HKG in a 30 min incubation period. Mg^{++} is not replaceable by Mn^{++} as a cofactor of oxidation.

Table IV

Enzymic ferricyanide-mediated oxidation of HKG by acetone powder of sweet potato mitochondria

	Initial cpm in 5-C^{14} HKG	cpm in malate	Initial cpm in 4-C^{14} HKG	cpm in malate
HKG + enzyme	34,320	230	14,400	120
" + Mg^{++} + enzyme	"	1383	"	664
" + TPP + enzyme	"	2813	"	1347
" + Mg^{++} + TPP + enzyme	"	3414	"	1750
As above, boiled enzyme	"	0	"	0

Reaction conditions: 3 μmoles HKG labeled as indicated, 80 μmoles phosphate pH 7.1, 1.8 μmoles $MgCl_2$, 5 μmoles potassium ferricyanide, 0.1 μmole TPP, mitochondrial acetone powder equivalent to 0.4 mg protein N. Final volume, 1 ml. Reaction time $\frac{1}{2}$ hr at room temperature. The reaction was stopped by boiling. Counts in malate were estimated following chromatographic isolation (see Materials and Methods).

Table V compares the HKG dehydrogenase activity of an extract of acetone powder of frozen Chlorella cells with the dehydrogenase activity towards several TCA cycle acids. The disparity between αKG dehydrogenase and HKG dehydrogenase is marked. The distinction is further affirmed in Table VI where the pH optima for αKG and HKG dehydrogenases are shown to be noticeably different.

Table V

The relative ferricyanide-mediated oxidation of several organic acids by an acetone powder extract of Chlorella

	ΔOD/625 mμ
malate	0.050
succinate	0.120
α-KG	0.030
pyruvate	0.030
HKG	0.160

Reaction conditions: substrate 6 μmoles, 80 μmoles phosphate, pH 7.1; 1.8 μmoles $MgCl_2$, 5 μmoles potassium ferricyanide, 0.1 μmole TPP in the case of the keto acids, and 1 μmole NAD in the case of malate. Enzyme equivalent to 6 mg acetone powder. Final volume 1 ml; reaction time $\frac{1}{2}$ hr at room temperature. The reaction was stopped by adding 0.1 ml of concentrated hydrochloric acid. Chlorella cells were stored in the deep freeze for a year.

Table VI

Comparison of pH optima for the enzymic ferricyanide-mediated oxidation of several α-keto acids

Substrate	pH 6.5	6.8	7.1	7.4	7.7	8.1
			ΔOD/625 mμ			
α-KG	0.850	0.780	0.650	0.470	0.330	0.140
Pyruvate	0.175	0.200	0.155	0.170	0.090	0.080
HKG	0.160	0.195	0.230	0.220	0.185	0.015

Reaction conditions as in Table V. The enzyme: acetone powder of sweet potato mitochondria equivalent to 0.2 mg protein N per reaction mixture.

Discussion

Two plant enzymes catalyze the oxidative decarboxylation of HKG to malate, an α-keto acid dehydrogenase and a peroxidase. The former enzyme was also found in rat liver mitochondrial preparations and in yeast. The indications are that HKG dehydrogenase is not identical with α-KG dehydrogenase since (a) there is no correlation between α-KG and HKG oxidizing capacity (Tables III and V); (b) the pH optima differ (Table VI); (c) freezing and thawing or prolonged freezing destroys the capacity to oxidize α-KG but has no effect on HKG oxidizing capacity (Tables III and V). It has been suggested that HKG metabolism proceeds via the cleavage of HKG to glyoxylate and pyruvate followed by oxidation of the cleavage products [Maitra and Dekker (1961) and Goldstone and Adams (1962)]. If such were the case one would expect $C^{14}O_2$ from the oxidation of 5-C^{14}-HKG and 4-C^{14}-HKG. When labeled HKG was oxidized by the preparations under study, there was practically no evolution of $C^{14}O_2$. Many reactions implemented by isolated enzyme systems have been reported capable of oxidizing glyoxylate, although their physiological occurrence in vivo is unestablished. The oxidation of glyoxylate to oxalate can be catalyzed by xanthine oxidase [Ratner et al. (1944) and Nakada and Weinhouse (1953)], by purified glycolic acid oxidase [Richardson and Tolbert (1961)], and by a CoA and NADP dependent glyoxylate dehydrogenase [Quayle and Taylor (1961)]. Nakada and Sund (1958) postulated a glyoxylate-glutamate pathway for the

oxidation of glyoxylate by rat liver mitochondria, in which a presumably nonenzymic interaction between the amino group of glutamate and the keto group of glyoxylate takes place followed by oxidative decarboxylation to yield formyl glutamate, which is hydrolyzed to formic acid and glutamate. Kornberg and Sadler (1960) proposed a dicarboxylic acid cycle for the oxidation of glycolate in which glyoxylate and acetyl CoA condense to form malate, the latter being oxidized with the ultimate regeneration of acetyl CoA. Malate synthatase is a prerequisite for such a cycle. Since malate synthatase is absent from animal tissue [Madsen (1958)] and is confined to a few higher plants [Yamamoto and Beevers (1960)] and microorganisms, the above pathway represents a special case. The enzymatic formation of HKG from glyoxylate and pyruvate has been demonstrated in animal tissue [Kuratomi and Fukunaga (1960)] and has recently been demonstrated by us in plant extracts. Since it has now been shown that HKG can be converted enzymatically to malate, which readily yields pyruvate, a new cycle for glyoxylate oxidation is proposed in which oxidation of glyoxylate depends on catalytic participation of pyruvate. In tissues containing glyoxylate-pyruvate lyase (HKG synthase) the oxidation of glyoxylate via an HKG cycle offers a likely metabolic path.

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