CONSTITUTIVE AND INDUCIBLE ISOZYMES OF α-KETOGLUTARIC SEMIALDEHYDE DEHYDROGENASE IN PSEUDOMONAS*

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In previous work (Adams, 1959; Yoneya and Adams, 1961; Adams and Norton, 1964; Singh and Adams, 1965a), we have described inducible enzymes of Pseudomonas striata that degrade hydroxyproline to α-ketoglutarate through the following reaction sequence:

L-hydroxyproline $\overset{1}{\rightleftarrows}$ D-allohydroxyproline $\overset{2}{\rightarrow}$ Δ^1-pyrroline-4-hydroxy-2-carboxylate $\overset{3}{\rightarrow}$ α-ketoglutaric semialdehyde $\overset{4}{\rightarrow}$ α-ketoglutaric acid

The last enzyme, α-ketoglutaric semialdehyde dehydrogenase, has recently been purified to or near homogeneity from sonic extracts of cells grown on L-hydroxyproline (Adams and Rosso, unpublished). Its difference in apparent inducibility from the preceding enzymes of this sequence led to the findings described here.

Enzymes 1, 2, 3 are undetectable or barely-detectable in extracts of cells grown in the absence of hydroxyproline and are estimated not to exceed 1% of their induced levels of activity. In contrast, after growth on a variety of substrates which do not induce the earlier enzymes, Pseudomonas extracts show considerable α-ketoglutaric semialdehyde dehydrogenase activity, ranging from 5% to 30% of the level induced by hydroxyproline. The dehydrogenase is also induced by growth on another substrate, D-glucarate, whose metabolism was recently shown by Dagley and Trudgill (1965) to involve the formation and

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oxidation of α-ketoglutaric semialdehyde. A study of the dehydrogenase in extracts of cells grown on L-proline (or other non-inducing substrates) and on hydroxyproline or D-glucarate showed that the non-induced and induced enzymes were closely similar in catalytic properties. The non-induced enzyme could be distinguished from the form induced by hydroxyproline or D-glucarate, however, by its different sedimentation and electrophoretic behavior.

METHODS

Cells of P. striata were grown, harvested and extracted by methods described in detail elsewhere (Yoneya and Adams, 1961). Hydroxyproline or other single amino acids were added as growth substrates at a concentration of 0.2%; when 0.2% glucose or glucarate or yeast extract (Difco) was the carbon source, 0.1% $(NH_4)_2SO_4$ was added to provide nitrogen. Crude enzyme, defined as the supernatant fluid of a sonic extract after centrifugation at 100,000 x g for 90 minutes, was used for all of the studies reported here except as noted. α-Ketoglutaric semialdehyde (KGSA) was prepared chemically as described earlier (Singh and Adams, 1965b); glutaric semialdehyde (GSA) was prepared by oxidation of α-aminoadipate with Chloramine-T as in a procedure used to degrade glutamate to succinic semialdehyde (Hendler and Anfinsen, 1954). Dehydrogenase activity was measured by the rate of formation of TPNH at pH 8.5 (pyrophosphate buffer) and 25°, the concentrations of aldehyde substrate and pyridine nucleotide being at least 10 times their K_m values. Units are μmoles of TPNH formed per minute.

RESULTS

Sedimentation and Electrophoretic Differences - Figure 1 shows the sedimentation behavior of purified enzyme induced by hydroxyproline, compared with crude enzyme similarly induced, and crude enzyme after constitutive growth conditions. It is evident that after growth on hydroxyproline, extracts contain two incompletely-separated sedimenting species of enzyme; the major peak is selectively enriched by purification, the minor peak (seen as a shoulder in Figure 1, B) sediments like the enzyme found after constitutive growth conditions.

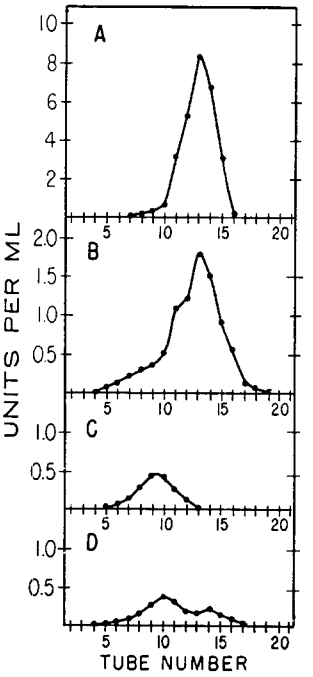

Fig. 1. Sedimentation of KGSA dehydrogenase through sucrose gradients. Units refer to KGSA as substrate, sedimentation was toward the left (Tube 1). The gradient was 20% to 5% in sucrose, containing 5 mM phosphate (pH 7.0), 5 mM reduced glutathione, and 1 mM sodium ethylenediamine tetraacetate. Approximately 0.3 mg of protein was used for A, approximately 5 mg of protein were used in each B, C, and D. (A) purified enzyme from hydroxyproline-grown cells; (B) crude enzyme from hydroxyproline-grown cells; (C) crude enzyme from proline-grown cells; (D) a 9:1 mixture of (C):(B). (B), (C) and (D) were centrifuged simultaneously in separate tubes of the same rotor; (A) was a separate run under similar conditions: 38,000 r.p.m., 16 hours, 5° C, in the SW-39 head of a Spinco Model L Centrifuge. Crude enzyme from glucarate-grown cells sedimented like (B) while enzyme from cells grown on each of the non-inducing substrates of Table I sedimented like (C). By the use of co-centrifuged enzymes (catalase and alcohol dehydrogenase) the molecular weight of the inducible form was estimated as 115,000 and that of the constitutive form about 50% greater.

In Figure 2, the two species are shown to be also separable by electrophoresis in starch gels, with the additional information that a trace of enzyme corresponding to the induced species can be demonstrated in crude extracts after growth under constitutive conditions.

Table I summarizes our finding that the major species of enzyme found in extracts of cells grown on a variety of non-inducing substrates has the properties illustrated in Figure 1 and 2 for enzyme from proline-grown cells, while

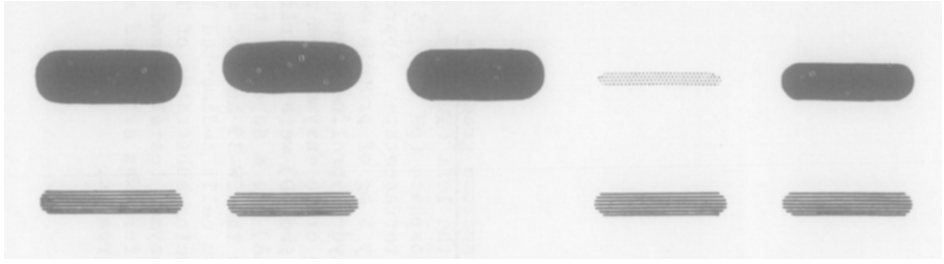

Fig. 2. Schematic representation, starch gel electrophoresis of KGSA dehydrogenase from cells grown on various substrates. 1, crude enzyme from L-hydroxyproline-grown cells; 2, crude enzyme from D-glucarate-grown cells; 3, purified enzyme from hydroxyproline-grown cells; 4, crude enzyme from L-proline-grown cells; 5, addition of 1, 2, or 3 to 4. Crude enzyme preparations from cells grown on the non-inducing substrates of Table I all gave patterns resembling 4. Vertical starch-gel electrophoresis was carried out for 4-6 hours in 0.025 M Tris buffer, pH 8.0, and enzyme bands were made visible by reaction with KGSA (or GSA) and TPN, followed by reduction of phenazine methosulfate and nitroblue tetrazolium. Enzyme bands migrated toward the anode from the origin-slots shown at the bottom of the figure.

the major species of enzyme induced by either hydroxyproline or glucarate differs from the constitutive types in a characteristic way. Table I also indicates the range of specific activity, with KGSA as substrate, in enzyme preparations after various growth conditions, as well as differences in the relative activity for KGSA and GSA, discussed below.

TABLE I

Some properties of KGSA dehydrogenase following growth on inducing substrates (hydroxyproline, glucarate) or non-inducing substrates

Carbon Source for Growth	Specific Activity* (units/mg protein)	Major Component		GSA / KGSA
		Electrophoresis	Sedimentation	
L-Hydroxyproline	50**	Fast	Slow	0.20
L-Hydroxyproline	0.98 (18)	"	"	0.23
D-Glucarate	0.91 (3)	"	"	0.27
L-Proline	0.14 (7)	Slow	Fast	1.0
L-Lysine	0.32 (2)	"	"	0.9
L-Glutamate	0.14 (2)	"	"	1.0
D-Glucose	0.07 (3)	"	"	1.0
Yeast Extract	0.11 (2)	"	"	1.1

* average values with KGSA as substrate; the number of determinations from separate cultures is shown in parentheses

** purified enzyme; all other values shown are for crude enzyme

Kinetic Comparisons - Since aldehyde dehydrogenases are widely distributed enzymes of differing substrate specificity, the initial observation of a high level of constitutive activity for KGSA oxidation was first considered likely to be due to an enzyme unrelated to induced KGSA dehydrogenase. Detailed comparisons were therefore made of the K_m values for KGSA, TPN, and DPN (a less active oxidant), of the ratio of activities with several other substrates (acetaldehyde, propionaldehyde and butyraldehyde), and of the pH-activity curves. These kinetic observations, which will be presented in detail elsewhere, revealed no convincing difference between the dehydrogenase activity

of crude preparations derived from proline-grown, hydroxyproline-grown or glucarate-grown cells, and led to the conclusion that the constitutive and induced enzymes are catalytically closely similar despite different size and electrophoretic properties. A comparison of purified and crude preparations of hydroxyproline-induced enzyme showed no significant difference in any of these measurements, supporting the validity of such comparisons for the unpurified enzymes.

Substrate Specificity - The enzyme purified from hydroxyproline-grown cells acts on a variety of aliphatic aldehydes from carbon-chain length three to ten. K_m values for these substrates were at least 100 times greater than for KGSA, while V_{max} did not exceed about 20% that for KGSA. Only one substrate tested, GSA, proved to be comparable in activity with KGSA, having a K_m of about 10^{-5} M. With this substrate a consistent kinetic difference was demonstrated between induced and constitutive extracts: at saturating concentrations of either substrate, GSA was oxidized only one-third to one-fifth as fast (relative to KGSA) by induced as by constitutive preparations (Table I). That the activity for GSA and KGSA were properties of the same enzymes was determined by starch gel electrophoresis; treating separate halves of the same starch slab with GSA or KGSA showed that only the two bands illustrated in Figure 2 reacted with GSA. Fractions from sucrose gradients or from Sephadex G-200 columns (which also permitted partial separation of the two peaks) were tested with both substrates; for both constitutive or induced enzyme preparations, aliquots representing the higher molecular weight species gave GSA/KGSA ratios of about 1, while those from the lower molecular weight region gave ratios of 0.5 to 0.3.

DISCUSSION

The foregoing observations indicate that Pseudomonas extracts contain two forms of KGSA dehydrogenase, closely similar in many catalytic properties but distinguishable by electrophoretic behavior, sedimentation rate and relative action on the substrates KGSA and GSA. One of these forms is present as the

major component in cell extracts grown on non-inducing substrates while the other component, present at trace levels in constitutive cells, is increased an estimated 50-fold or more after growth on either of two substrates, hydroxyproline or glucarate, which induce different sequences of degradative enzymes converging at the common step, α-ketoglutaric semialdehyde → α-ketoglutarate. It would clearly be of interest to examine the quantities of these two enzyme forms after growth on other substrates capable of inducing KGSA formation. The only other such inducer known (L-arabinose (Stoolmiller and Abeles, 1965)) or suspected (2-furoate (Singh and Adams, 1965b)), however, did not support growth as carbon sources alone and have not yet been tested as inducers when added to other growth substrates. The apparent identity of the dehydrogenase induced by either hydroxyproline or glucarate is tentatively explained by the hypothesis that KGSA, the immediate substrate of the enzyme, may itself be the inducer, although this possibility has not yet been tested conclusively.

The metabolic significance of a high constitutive level of one form of the enzyme is not clear. No evidence exists for the possible occurrence of KGSA as an intermediate in non-induced metabolic pathways. GSA, however, has been described as an intermediate in lysine degradation by another strain of Pseudomonas (Ichihara et al, 1960; Ichihara and Ichihara, 1961), and preliminary observations show that certain reactions of this pathway, lysine → δ-aminovalerate ⇄ GSA → glutarate, are demonstrable in extracts of our strain. It is thus possible that the major constitutive enzyme participates in a normal pathway for lysine degradation; a consistent observation is the relatively greater activity of this form of the enzyme for GSA than is shown by the inducible enzyme.

Many instances have been noted in recent years of the occurrence in the same cells of multiple forms of enzymes catalyzing a given reaction. Examples of isozymes with possible regulatory significance are the aspartokinases of E. coli that are individually repressible by separate end products (Stadtman et al, 1961; Freundlich, 1963); the alternate forms of the degradative enzyme, Δ^1-pyrroline dehydrogenase, separately induced in B. subtilis by arginine or proline

(de Hauwer et al, 1964); and the two forms of threonine deaminase (Umbarger and Brown, 1957) that were assigned degradative and biosynthetic roles respectively. The observations described here represent another example of isozymes with possible regulatory significance. In the present case, it appears that two catalytically similar forms of an enzyme are made constitutively. Either hydroxyproline or glucarate induces enzymes producing KGSA, and under these conditions one also observes apparent selective induction of the isozyme of KGSA dehydrogenase having preferential activity with KGSA as compared with GSA. Induction, rather than an activating effect of substrate on a pre-existing enzyme, is supported by the failure to demonstrate direct conversion of the constitutive to the inducible form by treatment of extracts with KGSA or glucarate. If one form of the enzyme is indeed selectively inducible, it would be expected that the two forms are proteins of different primary structure, and it should then be possible to obtain mutants in which one or the other isozyme is absent.

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