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Biochemistry

Activation of mouse epidermal tumor ornithine decarboxylase by GTP: Evidence for different catalytic forms of the enzyme

(enzyme regulation/chemical carcinogenesis/polyamine biosynthesis)

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ABSTRACT **In crude extracts of epidermal papillomas induced by an initiation–promotion protocol, ornithine decarboxylase (OrnDCase) activity was increased by the addition of GTP to the enzyme assay. No effect of GTP on the phorbol ester-induced enzyme isolated from normal epidermis was observed. Kinetic analyses indicated that the major effect of the nucleotide on the tumor-derived enzyme was to lower the apparent K_m for L-ornithine. When papilloma OrnDCase was partially purified by gel-filtration chromatography, two forms of the enzyme were resolved, only one of which was found in an epidermal extract from phorbol 12-myristate 13-acetate-treated mice. The enzymatic properties of the two forms of papilloma enzyme were compared. The higher molecular weight form (peak I) was activated by GTP, while the lower molecular weight form (peak II) was not. As expected from the kinetic analyses of the crude papilloma extracts, the apparent K_m of peak I enzyme for L-ornithine was very high (1.25 mM) but was much lower in the presence of GTP (0.02 mM). The two forms of papilloma OrnDCase differed in their sensitivities to heat inactivation and the ability of GTP to protect against heat inactivation. The $K_{1/2}$ for activation of peak I OrnDCase by GTP was 0.1 μM. The activation process was irreversible and did not require Mg^{2+}. When several nucleotides were tested for their ability to activate peak I OrnDCase, only GTP, dGTP, and the nonhydrolyzable derivative GTP[γ-S] were effective, while GDP, GMP, ATP, and CTP were relatively ineffective. Our results demonstrated the existence of two forms of OrnDCase in epidermal tumor extracts, of which one can be activated by GTP and one cannot. The significance of these findings for the regulation of this enzyme in normal and tumor cells is discussed.**

The ornithine decarboxylase (OrnDCase) present in benign mouse epidermal tumors (papillomas) is structurally and functionally different from the enzyme induced in normal mouse epidermis by the tumor promoter phorbol 12-myristate 13-acetate (PMA) (1). The enzymes isolated from these two sources differ in their heat stability, apparent K_m values for the substrate L-ornithine and cofactor pyridoxal 5'-phosphate, activation by the nucleotide GTP, and isoelectric points, as determined by two-dimensional immunoblots (1). Previous work has identified changes in the regulation of this gene in normal mouse epidermis vs. epidermis undergoing neoplastic development: in normal epidermis, the very low expression of this gene can be greatly increased by acute or chronic PMA treatment, but the increase in enzyme activity is transient (2). In epidermal tumors, however, this enzyme is constitutively expressed at high levels (3, 4). In view of the differences in the enzyme isolated from these two sources, it is possible that one explanation for the aberrant regulation of this gene in epidermal tumors is the presence of a structurally and functionally altered enzyme not subject to the normal regulatory mechanisms that control its synthesis and/or degradation.

One of the intriguing alterations in the OrnDCase of papillomas is its "activation"† by GTP. To our knowledge, such an activation has not been described for eukaryotic OrnDCase, but nucleotides including GTP are known to activate some bacterial OrnDCases (5). In a previous study, we ruled out bacterial contamination of the papillomas as an explanation for the effect of GTP (1). In the present study, we address the mechanism by which GTP increases the catalytic activity of the OrnDCase found in mouse epidermal tumors. Our results indicate that GTP activates OrnDCase activity chiefly by lowering the atypically high apparent K_m of the papilloma enzyme to a value characteristic of mammalian OrnDCase. Evidence is also presented that extracts of epidermal tumors, but not PMA-treated normal epidermis, contain two distinct forms of OrnDCase that differ markedly in their catalytic properties.

MATERIALS AND METHODS

Animals. Female CD1 mice were used, beginning at 7 wk of age. The dorsal hair was shaved at least 2 days prior to treatment and all chemicals were applied percutaneously to the shaved area. For papilloma induction, mice were initiated with a single treatment with 7,12-dimethylbenzanthracene, followed 1 wk later with twice weekly applications of 17 nmol of PMA. When at least some of the multiple tumors per animal reached a size of 5 mm, PMA treatment was stopped for at least 1 wk before the animals were killed and tumors were harvested for OrnDCase determinations. To obtain sufficient enzyme activity from epidermis, it was necessary to induce the enzyme with either a single or multiple (five) treatments with 17 nmol of PMA (3). In either case, mice were killed 4.5 hr later and epidermal extracts were prepared as described in the next section. The squamous cell carcinoma used (obtained from J. DiGiovanni, University of Texas System Cancer Center, Smithville, TX) was induced in a female Sencar mouse initiated with *N*-methyl-*N'*-nitrosoguanidine followed by biweekly promotion with PMA.

Preparation of Tumor Extracts. To harvest individual papillomas, mice were killed and tumors were quickly excised free of surrounding epidermis, weighed, wrapped in aluminum foil, and dropped in liquid N_2. The tumors were subsequently stored at −90°C until analysis. To prepare tumor extracts, the tumors were placed in 5 ml of ice-cold buffer A (25 mM Tris·HCl, pH 7.4, containing 2.5 mM

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Abbreviations: OrnDCase, ornithine decarboxylase; PMA, phorbol 12-myristate 13-acetate.

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†By activation is meant an increased activity of OrnDCase in a cell-free extract upon addition of GTP without implying a specific mechanism.

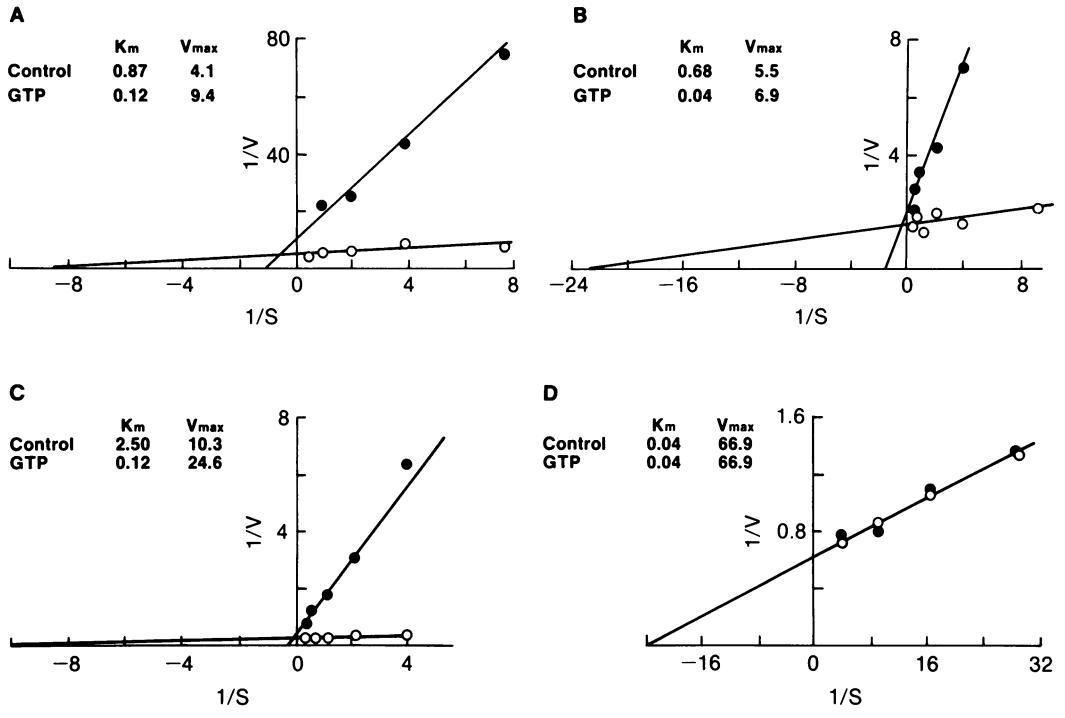

FIG. 1. The kinetics of activation of epidermal tumor OrnDCase by GTP. The enzyme activity of crude extracts of individual papillomas (*A* and *B*), a squamous cell carcinoma (*C*), or epidermis treated five times with PMA (*D*) was measured at various L-ornithine concentrations in the presence (○) or absence (●) of 0.1 mM GTP. The data are expressed as Lineweaver–Burk plots and the lines have been drawn by linear regression analysis. The units for the kinetic parameters are mM for K_m and units per mg of protein for V_{max}.

dithiothreitol and 0.1 mM EDTA) per g of tumor to which 0.5 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$) had been freshly added. The tumor was minced and homogenized in a Polytron homogenizer for two 15-sec bursts. The homogenate was then centrifuged at 20,000 × *g* for 20 min at 4°C. To the resulting supernatant, 1.5 g of solid $(NH_4)_2SO_4$ was added for each 10 ml, dissolved, and the solution was stirred for 30 min in the cold. After centrifugation, an additional 1.5 g of $(NH_4)_2SO_4$ per 10 ml of supernatant was added, dissolved, stirred for 30 min, and centrifuged. The resulting pellet was dissolved in a small volume of buffer A/$PhMeSO_2F$ and dialyzed overnight vs. 1000 vol of buffer A/$PhMeSO_2F$. Epidermal homogenates were prepared as described (2) except that buffer A/$PhMeSO_2F$ was used. Double $(NH_4)_2SO_4$ precipitation was done exactly as described for tumor extracts. The dialyzed tumor and epidermal extracts were stored at −90°C, with little or no loss of OrnDCase activity over a period of 3 mo. OrnDCase was assayed as described (6); 1 unit of activity is equivalent to 1 nmol of CO_2 produced per hr.

Chromatographic Procedures. An Ultrogel AcA 34 column (1.6 × 55 cm) was prepared and equilibrated with buffer B (buffer A/10 μM pyridoxal 5-phosphate/0.02% Brij 35). After loading of crude papilloma OrnDCase, elution was performed with the same buffer at a flow rate of 5 ml/hr, collecting 1-ml fractions. GTP agarose chromatography was performed by incubation of crude tissue extracts with 2 ml of affinity matrix in 4 ml of buffer C (10 mM Tris·HCl/90 mM NaCl/1 mM EDTA/2.5 mM dithiothreitol/0.02% Brij 35, pH 7.4). After gentle mixing for 2 hr at 4°C, the mixture was poured into a column and washed with 6 column vol of buffer C followed by 2 column vol of buffer C + 0.1 mM GTP. The GTP-eluted fractions were extensively dialyzed (vs. 1000 vol of buffer C, with two changes) prior to OrnDCase assay.

Sources. Chemicals used and their sources are as follows: PMA (Chemicals for Cancer Research, Eden Prairie, MN);

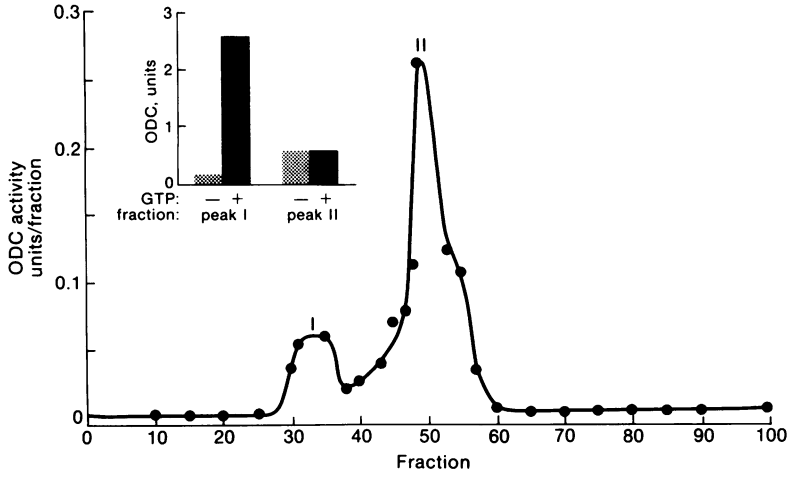

FIG. 2. Ultrogel AcA 34 chromatography of papilloma OrnDCase (ODC). A crude extract derived from a single papilloma was chromatographed as described in *Materials and Methods*. Fraction 1 is taken as the first fraction after the void volume of the column. After initial assays to determine active fractions, fractions 30–35 (peak I) and 46–49 (peak II) were pooled and concentrated ≈10-fold using Centricon-30 miniconcentrators (Amicon). The pooled concentrated peak fractions were then assayed for OrnDCase in the presence or absence of 0.1 mM GTP at an L-ornithine concentration of 0.125 mM (*Inset*).

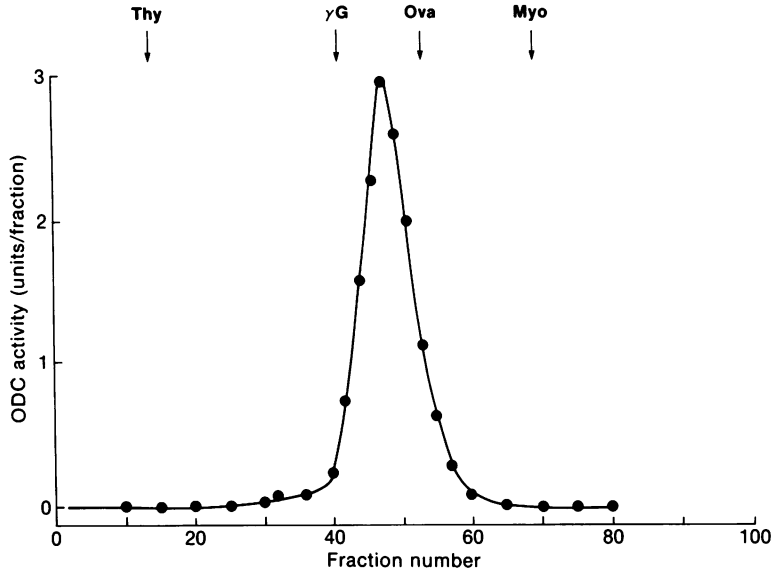

FIG. 3. Ultrogel AcA 34 chromatography of epidermal OrnDCase. A crude extract of epidermis derived from mice treated five times with 17 nmol of PMA was chromatographed on the same column used for the experiment shown in Fig. 2. Some of the peak fractions containing OrnDCase (ODC) activity were also assayed in the presence of 0.1 mM GTP. No effect was observed (data not shown). The elution positions of standard proteins (molecular weights in parentheses) are indicated: Thy, thyroglobulin (670,000); γG, immunoglobulin (158,000); Ova, ovalbumin (44,000); and Myo, myoglobin (17,000).

guanine nucleotides (Sigma); Ultrogel AcA 34 (LKB); GTP agarose (Pharmacia).

RESULTS

Characteristics of GTP Activation of Epidermal Tumor OrnDCase. Assays of OrnDCase activity in crude extracts of several individual mouse epidermal papillomas in the presence or absence of 0.1 mM GTP at a single ornithine concentration (usually 0.25 mM) indicated that the nucleotide caused an increase of variable magnitude in enzyme activity (ref. 1; data not shown). To further characterize this activation, the effect of this nucleotide on epidermal tumor OrnDCase was determined at several L-ornithine concentrations. As shown in Fig. 1, GTP caused a substantial decrease in the apparent K_m for L-ornithine in three extracts of individual epidermal tumors (two papillomas, one squamous cell carcinoma), while having no effect on the apparent K_m of PMA-induced mouse epidermal OrnDCase. In similar experiments with two other tumor extracts, GTP always caused a similar shift in the apparent K_m for L-ornithine (data not shown) from values higher than those usually found for mammalian OrnDCase (i.e., 0.2–2 mM) to values more typical of the K_m of PMA-induced epidermal OrnDCase. If the results of experiments on five different tumor extracts are combined, the mean K_m for L-ornithine in the presence of GTP [0.076 ± 0.013 mM (n = 5); range, 0.04–0.12] was not significantly different than that for PMA-induced epidermal enzyme (0.07 mM; see ref. 1). An effect of GTP was also seen on the V_{max} of tumor OrnDCase (Fig. 1). However, while a reduction in apparent K_m for L-ornithine in the presence of GTP was observed in every tumor extract examined, the V_{max} effect was more variable, including a lack of effect in some cases.

Chromatographic Analysis of Papilloma vs. Epidermal OrnDCase. Although previous work had identified several differently charged forms of both epidermal and papilloma OrnDCase in the isoelectric focusing dimension of denaturing two-dimensional gels (1), the question remained whether different forms of catalytically active OrnDCase could be resolved under nondenaturing conditions. When a crude extract of a single papilloma was applied to an Ultrogel AcA 34 gel filtration column, two peaks of OrnDCase activity were found in the eluted fractions (Fig. 2). The active fractions comprising each peak were then pooled, concentrated, and assayed in the presence or absence of GTP. As shown in Fig. 2 (*Inset*), only the enzyme present in peak I was activated by GTP. This experiment has been repeated with two other papilloma extracts with identical results: only the higher molecular weight peak of enzyme activity was activated by GTP while the lower molecular weight peak of activity was unaffected by GTP (data not shown). When a crude epidermal extract derived from the PMA-treated mice was applied to the same column (Fig. 3), only one peak of enzyme activity was observed, which corresponded to peak II of the papilloma extract. To compare the enzymatic properties of peak I and peak II OrnDCase, several papillomas were pooled and chromatographed on Ultrogel AcA 34 to obtain sufficient OrnDCase activity. The peak I and peak II OrnDCases thus isolated were used for the following series of experiments. As expected because of the data illustrated in Fig. 1, the apparent K_m for L-ornithine of peak I OrnDCase was 1.25 mM but was reduced to 0.02 mM in the presence of 0.1 mM GTP. The apparent K_m for L-ornithine of peak II OrnDCase was 0.05 mM and it was not affected by GTP. When peak I OrnDCase was assayed in the presence of different concentrations of GTP (Fig. 4), the $K_{1/2}$ for activation was ≈0.1 μM. Once exposed to GTP, the activation of peak I OrnDCase was apparently irreversible—despite extensive dialysis, the enzyme remained activated and was only slightly stimulated by readdition of GTP (Table 1). However, experiments using [^{3}H]GTP in similar experiments have shown that a significant

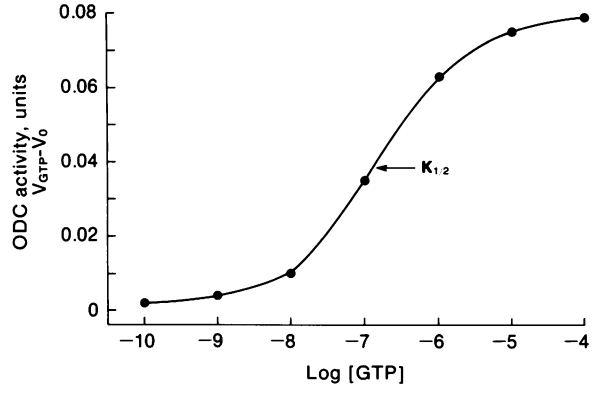

FIG. 4. The concentration dependence of GTP activation of papilloma OrnDCase (ODC). Partially purified OrnDCase (peak I; see Fig. 2) was incubated with the indicated concentration of GTP for 5 min at 0°C and then assayed for OrnDCase activity at 37°C at a substrate concentration of 0.25 mM. Results are expressed as the increase in enzyme activity due to the presence of GTP (i.e., $V_{GTP} - V_o$, where V_o = 0.01 unit). Points are the average of duplicate determinations.

Table 1. Is the activation of peak I OrnDCase by GTP reversible

Preincubation	GTP in assay	OrnDCase activity Unit	+ GTP/ − GTP
H_2O	−	0.007	7.9
	+	0.055	
GTP (0.1 mM)	−	0.075	1.3
	+	0.096	
GTP (0.1 mM) + $MgCl_2$ (10 mM)	−	0.089	1.1
	+	0.097	

Identical aliquots of peak I OrnDCase were preincubated with GTP, GTP plus $MgCl_2$, or nothing for 10 min at 37°C, followed by extensive dialysis (2000 vol of buffer A with three changes) for 24 hr at 4°C. $MgCl_2$ was included in the dialysis buffer for the sample preincubated with GTP plus $MgCl_2$. OrnDCase activity was subsequently assayed in the presence and absence of GTP and the -fold stimulation of enzyme activity by GTP was calculated (+GTP/−GTP). The experiment has been repeated with another preparation of peak I OrnDCase with similar results.

fraction of GTP is resistant to dialysis under these conditions (T.G.O. and K.O., unpublished data). The activation process did not require Mg^{2+} (Table 1). The specificity of activation of peak I OrnDCase was tested by incubation of the partially purified enzyme with various nucleotides, all at 1 μM (Table 2). The most effective nucleotides were GTP, dGTP, and the nonhydrolyzable derivative (GTP[γ-S]). GDP was slightly active but GDP[β-S] was not, suggesting either the GDP used might have been contaminated with GTP or metabolism to GTP had occurred during the enzyme assay. The other nucleotides tested (GMP, ATP, CTP) were not active in the assay. Thus, in terms of $K_{1/2}$, irreversibility, and nucleotide specificity, the activation of epidermal tumor OrnDCase is similar in many respects to other GTP-dependent enzyme systems (7).

Since we have previously found that crude extracts of epidermal tumor OrnDCase and epidermal OrnDCase differ in their sensitivity to heat inactivation (1), it was of interest to compare the heat sensitivities of partially purified peak I vs. peak II OrnDCase in the presence and absence of GTP. As shown in Fig. 5, the presence of GTP had a substantial protective effect against heat inactivation of peak I OrnDCase but had little effect on peak II OrnDCase. In other experiments (data not shown), the heat-inactivation profile of partially purified epidermal OrnDCase (Fig. 3) was unaffected by GTP.

Since epidermal tumors contain at least one form of OrnDCase capable of binding GTP with high affinity, the ability of tumor OrnDCase to bind to GTP agarose was

Table 2. Activation of papilloma OrnDCase by various nucleotides

Addition	OrnDCase activity Unit	% of control
None	0.016	100
GTP	0.120	750
GDP	0.033	206
dGTP	0.083	519
GTP[γ-S]	0.108	675
GDP[β-S]	0.019	120
GMP	0.013	81
CTP	0.013	81
ATP	0.012	75

Equal amounts of papilloma peak I OrnDCase were incubated with the indicated nucleotide (or H_2O) at 1 μM for 5 min at 0°C. Enzyme activity was then measured at 37°C for 60 min at an L-ornithine concentration of 0.125 mM. Values are the average of duplicate determinations.

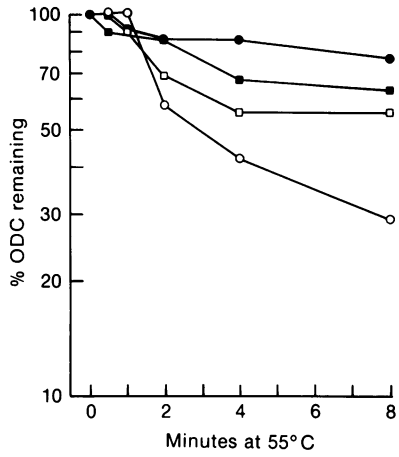

FIG. 5. Effect of GTP on heat inactivation of partially purified papilloma OrnDCase(s) (ODC). Aliquots of peak I (○ and ●) and peak II (□ and ■) OrnDCase from Ultrogel AcA 34 chromatography (Fig. 2) were incubated at 55°C in the presence (solid symbols) or absence (open symbols) of 0.1 mM GTP. Aliquots were removed at the indicated times to precooled tubes at 0°C and subsequently assayed for OrnDCase activity. Results are expressed as percentage of OrnDCase activity remaining relative to nonincubated controls.

determined. As shown in Fig. 6, a portion of the tumor OrnDCase applied to a GTP agarose column bound and could be eluted with GTP. The OrnDCase eluted from the column by GTP was irreversibly activated since, despite extensive dialysis, addition of GTP had no effect on enzyme activity (data not shown). This result is consistent with the data presented in Table 1, demonstrating the irreversibility of activation of peak I OrnDCase by GTP. None of the epidermal OrnDCase bound to the affinity matrix under the same conditions used for the tumor enzyme.

DISCUSSION

Unlike eukaryotic OrnDCases isolated from other mammalian sources, the OrnDCase activity present in chemically induced mouse skin tumors can be activated by GTP (1). In this report, we demonstrate that this activation is due to a large decrease in the apparent K_m of the enzyme for L-ornithine as well as, in some cases, an increase in the V_{max} of the enzyme. We have previously reported that the enzymatic properties of OrnDCase from all mouse epidermal papillomas

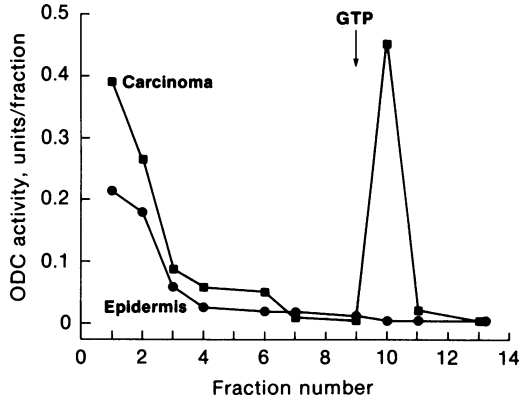

FIG. 6. GTP agarose chromatography of epidermal vs. tumor OrnDCase (ODC). Crude extracts of PMA-induced epidermal OrnDCase or a single carcinoma were chromatographed as described in *Materials and Methods*. After elution with GTP and extensive dialysis, all fractions were assayed for OrnDCase activity. Similar results have been obtained for two other individual tumor extracts and one other preparation of epidermal OrnDCase.

examined are different from the enzyme isolated from phorbol ester-treated mouse epidermis (1). One of the striking differences found was the much higher K_m values of individual papilloma OrnDCases for L-ornithine (0.28–1.2 mM) compared to the epidermal enzyme (0.07 mM). In light of the data in this study, these higher apparent K_m values are likely to be physiologically irrelevant since in the intact cell with sufficient GTP present, the K_m values are similar to the epidermal enzyme (0.076 ± 0.013 mM; mean ± SEM; $n = 5$). Given that the $K_{1/2}$ for GTP activation is ≈0.1 μM (Fig. 4), it is unlikely that GTP is limiting *in vivo* and, thus, the enzyme may always be in an activated state. The reason why an effect of GTP can be demonstrated in crude tumor extracts is that the $(NH_4)_2SO_4$ fractionation procedure can apparently release bound GTP from the tumor enzyme allowing "reactivation" of the enzyme by GTP (T.G.O. and O.H., unpublished data). This contrasts with the apparent (but not real) irreversible activation of peak I OrnDCase shown in Table 1, which simply attests to the tight binding of GTP to OrnDCase or another effector protein that is resistant to dissociation during dialysis. In contrast to the properties of the tumor OrnDCase, the enzyme present in (induced) normal epidermis has a typically low apparent K_m for L-ornithine and is not activated by GTP (1).

While it is clear that GTP alters the catalytic activity of epidermal tumor OrnDCase by "normalizing" the unusually high apparent K_m for L-ornithine (Fig. 1), the exact molecular details involved are not clear. At least two mechanisms are possible. First, GTP could bind to tumor OrnDCase directly and affect its catalytic function. Second, the effect of GTP could be mediated by a GTP-binding protein that upon activation by GTP binds to OrnDCase and alters its activity. Evidence in favor of the latter hypothesis is the existence in tumor extract of two catalytically active molecular weight forms of OrnDCase, with only the higher molecular weight form susceptible to activation by GTP (Fig. 2). However, attempts to dissociate a putative complex between OrnDCase and an effector protein, such as by chromatography in high salt buffers, have been unsuccessful (O.H. and T.G.O., unpublished data). To decide this question, it will be necessary to purify to homogeneity the OrnDCases from mouse tumors to determine the exact mechanism of GTP activation. If during purification the ability of GTP to activate peak I OrnDCase is lost, a GTP-binding protein would be implicated and could be assayed for by reconstitution of purified OrnDCase plus the putative effector molecule in the presence and absence of GTP. Alternatively, if GTP activation is not lost during purification, a direct mechanism involving GTP binding to OrnDCase itself would be implicated. Such studies are necessary but are hampered by the very low content of OrnDCase in epidermal tumors (1–10 ppm of the soluble protein; T.G.O., unpublished data).

The mechanism(s) responsible for the appearance of altered forms of epidermal tumor OrnDCase is unknown. Since carcinogen treatment is used to initiate the process of tumor development in this system, it is possible that a carcinogen-induced mutation accounts for the altered properties of the enzyme. Alternatively, since OrnDCase is potentially a multigene family in rodents (8, 9), papillomas might express a previously "silent" member of the family. In addition, differences in posttranslational modification, either alone or in combination with one of the mechanisms described above, could account for our results. Finally, although considered unlikely, contamination of tumor extracts with bacterial OrnDCase might conceivably explain our results.‡ Molecular cloning and sequencing of the OrnDCase cDNAs isolated from the appropriate epidermal and papilloma libraries should illuminate the actual molecular mechanisms involved.

Whatever the mechanisms responsible, the significance of a "different" OrnDCase in epidermal tumors derived by an initiation–promotion protocol for the regulation of this enzyme *in vivo* is not clear. Older evidence implicating a longer half-life of the enzyme in papillomas (11) as well as recent data from this laboratory demonstrating the "constitutive" expression of high levels of OrnDCase protein (4) and its mRNA (12) in epidermal tumors compared to normal or promoter-treated epidermis strongly suggest that this gene product is regulated differently in tumors than in normal epidermis. Our working hypothesis is that the altered enzyme structure of at least some form(s) of papilloma OrnDCase renders the enzyme not susceptible to the normal cellular regulation of this protein. If so, it is possible that some of the unique properties of this enzyme, such as its extremely short half-life *in vivo*, may be lost in epidermal tumors. The consequences of a constitutively expressed high level of OrnDCase in a preneoplastic cell are not known but combined with other genetic and epigenetic changes might contribute to the further progression of such a cell toward malignancy.

‡The peak I OrnDCase, present in epidermal tumors, has some of the properties of bacterial OrnDCase, such as activation by GTP (5) and a resistance to irreversible inactivation by the ornithine analog α-difluoromethylornithine (ref. 10; T.G.O. and O.H., unpublished results). However, there are many other experimental observations of ours and others (11) that argue strongly against bacterial contamination of tumor extracts, including differences in apparent values for L-ornithine of the two enzymes, the extreme heat lability of bacterial OrnDCase vs. the relative resistance of peak I OrnDCase to heat inactivation, the inability of Polytron homogenization to liberate OrnDCase from bacterial suspensions, and the complete absence of the bacterial enzyme arginine decarboxylase from crude tumor extracts (O.H. and T.G.O., unpublished data).

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