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Subunit Structure of the Orotate Phosphoribosyltransferase–Orotidylate Decarboxylase Complex from Human Erythrocytes†

Garry K. Brown and William J. O'Sullivan*

ABSTRACT: A complex of orotate phosphoribosyltransferase and orotidylate decarboxylase has been shown to exist in three molecular weight forms (Brown, G. K., Fox, R. M., and O'Sullivan, W. J. (1975), *J. Biol. Chem. 250,* 7352). The smallest of these, of molecular weight 62 000, was subjected to further study. On the basis of the inactivation of the enzyme activities, carried out in the presence of low concentrations of guanidine hydrochloride, and of changes in molecular weight of preparations during aging, it was inferred that the enzyme complex contained more than one type of subunit. This was confirmed by chromatography on Sephadex G-75 after preincubation in guanidine hydrochloride or with guanidine hydrochloride in the elution buffer. It was concluded that the enzyme complex consisted of two types of subunits, two decarboxylase units of molecular weight approximately 20 000 and two further subunits of approximately 13 000. The subunits could be separated and reassociated with partial recovery of both activities. A 40 000 molecular weight form had full decarboxylase activity but no phosphoribosyltransferase activity. Restoration of the 62 000 molecular weight form resulted in restoration of both enzymatic activities. An intermediate species of molecular weight 50 000 representing a combination of the decarboxylase dimer with one of the 13 000 subunits was also demonstrated. This form required the presence of dithiothreitol in order to manifest phosphoribosyltransferase activity. A model of the system has been proposed that accounts for both the different molecular weight forms and also for the deficiency of both activities in the rare inborn error of metabolism, hereditary orotic aciduria.

Hereditary orotic aciduria is a rare inborn error of pyrimidine metabolism in man. It is usually characterized by marked deficiency of two sequential enzymes of the de novo pyrimidine biosynthetic pathway, orotate phosphoribosyltransferase (EC 2.4.2.10) (reaction 1) and orotidylate decarboxylase (EC 4.1.1.23) (reaction 2) (Smith et al., 1972).

$$\text{orotate} + \text{PRibPP}^{\text{l}} \xrightarrow{Mg^{2+}} \text{OMP} + \text{PP} \qquad (1)$$

$$\text{OMP} \rightarrow \text{UMP} + CO_2 \qquad (2)$$

Two variant forms of the disease have also been described, one lacking only orotidylate decarboxylase (Fox et al., 1969), the other having a selective deficiency of orotate phosphoribosyltransferase (Worthy et al., 1974). The two enzymes appear to behave in a coordinated fashion in all mammalian tissues in which they have been studied. Copurification of the enzymes has been observed by a number of investigators, subject to the limitations imposed by the greater instability of the phosphoribosyltransferase (Kasbekar et al., 1964; Appel, 1968; Brown et al., 1975; Grobner and Kelley, 1975). Also, the ratio of the two activities was found to be constant over a wide range in fresh preparations of human hemolysate (Fox et al., 1971). This coordinate behavior of the two enzymes led to the suggestion that the genes coding for them could be linked and that hereditary orotic aciduria could arise from a defect in a regulator gene or that the two enzyme activities might reside

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on a single polypeptide chain (Smith et al., 1972). Support for a possible regulator gene defect in hereditary orotic aciduria was provided by the experiments of Pinsky and Krooth (1967), who found that mutant fibroblasts from patients with hereditary orotic aciduria grown in tissue culture with an inhibitor of orotidylate decarboxylase showed an increase in the specific activity of both enzymes to near normal levels.

Recently, the orotate phosphoribosyltransferase-orotidylate decarboxylase complex in human erythrocytes was shown to exist in a number of molecular weight forms which differed in physical and kinetic properties (Brown et al., 1975; Grobner and Kelley, 1975). The structure of the smallest molecular weight form (62 000) of the complex observed in this laboratory has now been further studied. In this paper, experiments are described which demonstrate that this form consists of smaller subunits associated with orotate phosphoribosyltransferase and orotidylate decarboxylase activities, respectively. The subunits can be separated but appear to be inactive alone; however, activity can be partly restored if the subunits are recombined. Various combinations of the subunits can occur and these differ in their properties with respect to one or both enzymes. A model for the genetic defects in the various forms of hereditary orotic aciduria can be described in terms of the substructure of the basic unit of the orotate phosphoribosyltransferase-orotidylate decarboxylase complex.

Materials and Methods

Materials. [*carboxyl*-^{14}C]Orotic acid and [*carboxyl*-^{14}C]orotidine 5′-monophosphate (OMP)[1] were purchased from New England Nuclear Corp.; orotic acid, tetrasodium 5′-phosphoribosyl 1′-pyrophosphate (PRibPP) and OMP were from Sigma Chemical Co.; dithiothreitol, crystalline human serum albumin, pepsin, and hemoglobin were from Calbiochem Pty. Ltd. DEAE-cellulose was obtained from Whatman Biochemicals Ltd. and Sephadex G-75 and G-100 and CM-Sephadex G-50 were from Pharmacia Fine Chemicals. Glyceraldehyde-phosphate dehydrogenase (from rabbit muscle) and 3-phosphoglycerate kinase (from yeast) were prepared by the methods of Scopes (1969, 1971). Guanidine hydrochloride was obtained from Schwarz/Mann.

Enzyme Assays. Orotate phosphoribosyltransferase and orotidylate decarboxylase were estimated by the release of $^{14}CO_2$ from [*carboxyl*-^{14}C]orotic acid and [*carboxyl*-^{14}C]-OMP as previously described (Brown et al., 1975; Fox et al., 1971; Fox, 1971).

Orotate Phosphoribosyltransferase Assay. The reaction mixture contained Tris-HCl, pH 7.4, 50 mM; [*carboxyl*-^{14}C]orotic acid, 0.2 mM (25 nCi); $MgCl_2$, 0.3 mM, and PRibPP, 0.25 mM, in a total volume of 1.0 mL. Under normal conditions, sufficient endogenous orotidylate decarboxylase was present to convert all OMP formed to UMP. When these conditions did not apply, 0.1 unit of yeast orotidylate decarboxylase, prepared by the method of Umezu et al. (1971) and free of orotate phosphoribosyltransferase activity, was added to the reaction mixture.

Orotidylate Decarboxylase Assay. The standard reaction mixture contained 50 mM Tris-HCl, pH 7.4, and 0.08 mM [*carboxyl*-^{14}C]OMP (7 nCi) in a total volume of 1.0 mL. The protein concentration in all assays was greater than 0.5 mg/mL. In cases where the enzyme sample did not contain this amount, it was brought to 0.5 mg/mL with human serum albumin.

Protein Determination. Protein was estimated by the method of Goodwin and Choi (1970).

Enzyme Purification. The method described by Hatfield and Wyngaarden (1964) for beef erythrocytes was used as a basis to prepare orotate phosphoribosyltransferase and orotidylate decarboxylase from human erythrocytes. The procedure was substantially as previously reported from this laboratory (Brown et al., 1975) except that two variations were introduced. Erythrocytes were obtained from patients with secondary polycythaemia (usually due to cyanotic congenital heart disease) at Royal Prince Alfred Hospital. They were separated from white blood cells and plasma by spinning at 1200*g* for 30 min followed by three washes in 0.155 M KCl, all at 4 °C. After each spin, the cells to approximately 1 cm below the buffy coat were discarded to minimize any contamination. The cells were lysed by suspension in 5 mM potassium phosphate, pH 7.4, and the cell membranes were removed by centrifugation. The hemolysate was stirred with DEAE-cellulose preequilibrated with 10 mM potassium phosphate, pH 7.4. The unbound hemoglobin was removed by washing on a Büchner funnel and the DEAE-cellulose was poured into a column. This was washed with 10 mM potassium phosphate, pH 7.4, until the optical density at 280 nm of the emerging buffer was less than 0.1. The enzymes were eluted with a linear gradient of potassium phosphate, pH 7.4, from 10 to 300 mM. The fractions containing the peak of enzyme activities were pooled and brought to 95% saturation with solid ammonium sulfate. The precipitate was collected by centrifugation and dilayzed against 50 mM potassium phosphate, pH 7.4.

At this stage two variations were introduced. In the first, the enzyme preparation from the DEAE-cellulose step was applied to a Sephadex G-150 column (1.5 × 80 cm) and eluted with 50 mM potassium phosphate, pH 7.4. This step separated the decarboxylase activity into three parts which corresponded to molecular weights of 62 000, 115 000 and 250 000, respectively.

In the second variation, the enzyme preparation was stirred with CM-Sephadex C-50 that had been equilibrated with 10 mM potassium phosphate, pH 6.6, and poured into a column (1.5 × 30 cm). The column was washed with the same buffer until all unbound protein had been washed out. The enzymes were then eluted over a linear gradient of potassium phosphate, pH 6.8, from 10 to 250 mM. Fractions containing enzyme activity were identified by assay, pooled, and brought to 95% saturation with ammonium sulfate. The precipitate was collected by centrifugation and dialyzed against 50 mM potassium phosphate, pH 7.4.

Sephadex Chromatography. Separation of different molecular weight forms of both enzymes was achieved using 40 × 1.0 cm columns of Sephadex G-75 and G-100 depending on the molecular weight species being studied. The column total volume was 30 mL and the flow rate 10 mL/h.

The Sephadex columns were calibrated with the following proteins of known molecular weight: glyceraldehyde-phosphate dehydrogenase (140 000), hemoglobin (67 000), human serum albumin (66 000), 3-phosphoglycerate kinase (46 000), pepsin (30 000), and soybean trypsin inhibitor (21 500).

Effect of Guanidine Hydrochloride. The inactivation of both enzymes by guanidine hydrochloride was studied by incubating the enzyme with this compound for various periods of time at 4 °C. At each time interval, an aliquot was withdrawn and diluted into the assay mixtures so that the final

[1] Abbreviations used are: OMP, orotidine 5′-monophosphate; PRibPP, 5′-phosphoribosyl 1′-pyrophosphate; DEAE, diethylaminoethyl; CM, carboxymethyl; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; EDTA, (ethylenedinitrilo)tetraacetic acid.

TABLE I: Purification of Orotate Phosphoribosyltransferase and Orotidylate Decarboxylase from Hemolysate.

Fraction	Vol (mL)	Protein (mg/mL)	Sp Act. (nmol mg^{-1} h^{-1})		Yield (%)	Purification
			OPRTase	ODCase		
Hemolysate	1066	105	0.15	0.30	100	
DEAE-cellulose:$(NH_4)_2SO_4$ ppt	31	11	49	90	86	230
CM-Sephadex:$(NH_4)_2SO_4$ ppt	6.4	3.4	59 (110[a])	264	20	880

[a] Assayed in the presence of 1 mM dithiothreitol and 10 μM EDTA.

guanidine concentration was too low to interfere with the assays.

In some experiments, the enzyme preparation that had been preincubated at various guanidine hydrochloride concentrations was divided into two batches. One batch was eluted from Sephadex G-75 with potassium phosphate buffer (50 mM, pH 7.4) alone, the other with guanidine hydrochloride, at the same concentration as that in which it had been preincubated, in addition to the phosphate buffer.

Results

Enzyme Purification. Enzyme prepared by two procedures was used in the studies reported in this paper. The first procedure (preparation 1) varied only slightly from that used in a previous report (Brown et al., 1975). This preparation represented a 400-fold purification over the original hemolysate. The relative activities of both the orotate phosphoribosyltransferase and orotidylate decarboxylase remained constant in the ratio of approximately 1:2 as observed previously (Fox et al., 1971). It was resolved into different forms on Sephadex G-150 and the lowest molecular weight form (ca. 62 000) was used for further investigation.

The introduction of a CM-Sephadex step (preparation 2) produced a greater degree of purification (880-fold) of the decarboxylase but an apparent relative loss of the phosphoribosyltransferase activity (Table I). The latter activity could be effectively restored by assaying in the presence of thiol (viz. 1 mM dithiothreitol, 10 μM EDTA), which, however, had no discernible effect on the decarboxylase. This procedure appeared to yield entirely the low-molecular-weight form (62 000) of the complex but subsequent studies indicated that a lower molecular weight species (~50 000) was also present.

Subunit Structure of the Phosphoribosyltransferase-Decarboxylase Complex. Evidence that the 62 000 molecular weight form of the orotate phosphoribosyltransferase-orotidylate decarboxylase complex contained more than one subunit was obtained, firstly, from molecular weight changes associated with changes in activity on storage of the enzymes, and, secondly, from observations on the differential inactivation of the two enzyme activities in the presence of guanidine hydrochloride. These observations were supported by separation of various enzymatically active forms by Sephadex gel filtration following incubation of the complex with guanidine hydrochloride and by subsequent recombination of the fractions to restore enzyme activity.

(A) Effect of Storage on Enzyme Activity. Prolonged storage (>1 month) of enzyme (preparation 1) at −20 °C in 50 mM potassium phosphate, pH 7.4, had little effect on decarboxylase activity but the specific activity of the orotate phosphoribosyltransferase progressively decreased. The decrease occurred in two stages. Firstly, the activity which was measurable with orotic acid and Mg-PRibPP alone decreased with a half-life of approximately 6 weeks. During this period almost all of the initial orotate phosphoribosyltransferase activity was recoverable if dithiothreitol and EDTA were included in the assay mixture. When the activity of orotate phosphoribosyltransferase detectable with substrates alone fell to ~10% of the initial activity, the activity which was recoverable with dithiothreitol began to decline with a half-life of about 1 week.

A similar pattern of inactivation was seen with enzymes prepared by the CM-Sephadex procedure (preparation 2) described above. This preparation usually had most of the orotate phosphoribosyltransferase in the form which was only active in the presence of dithiothreitol and the activity was quite unstable under the storage conditions used. Again, the specific activity of the decarboxylase decreased only slightly during storage and was almost unaffected by the addition of dithiothreitol to the assay mixture. Any changes in orotidylate decarboxylase specific activity with dithiothreitol appeared to be related to the form of orotate phosphoribosyltransferase present in the enzyme sample. The greatest effect was observed when a large fraction of the phosphoribosyltransferase activity was only measurable in the presence of dithiothreitol.

Alteration of the 62 000 Molecular Weight Form of the Complex during Storage. The molecular weight distribution of the phosphoribosyltransferase-decarboxylase complex under different conditions was further studied by using samples of both enzyme preparations stored for various periods. The samples included those in which the phosphoribosyltransferase activity as measured with substrates alone was 50% of the decarboxylase activity, those in which a fraction of the phosphoribosyltransferase activity could only be measured in the presence of dithiothreitol (aged preparation 2), and a sample of enzyme (preparation 1) stored for 6 months which had a high level of decarboxylase activity but no detectable phosphoribosyltransferase activity either alone or with dithiothreitol.

The elution profiles of these enzymes on Sephadex G-75 are shown in Figure 1. A fresh sample of preparation 1, containing the normal ratio of the two enzymes, eluted as a single peak containing both activities (Figure 1(I)). A sample of preparation II, containing 30% of the transferase measurable with substrates alone and the remaining 70% recoverable with dithiothreitol (Figure 1(II)) showed a single peak of decarboxylase activity but two peaks of phosphoribosyltransferase activity. The presence of two peaks of activity was consistently observed in more than 20 experiments with different enzyme preparations and different sized columns. The position of the *slower*-moving component represented a decrease of 12 000–13 000 in molecular weight to yield a species of approximate molecular weight of 50 000. The elution profile of the sample of aged preparation I containing only decarboxylase activity (Figure 1(III)) is also shown. This enzyme eluted as a single peak with a molecular weight of ~35 000. The results

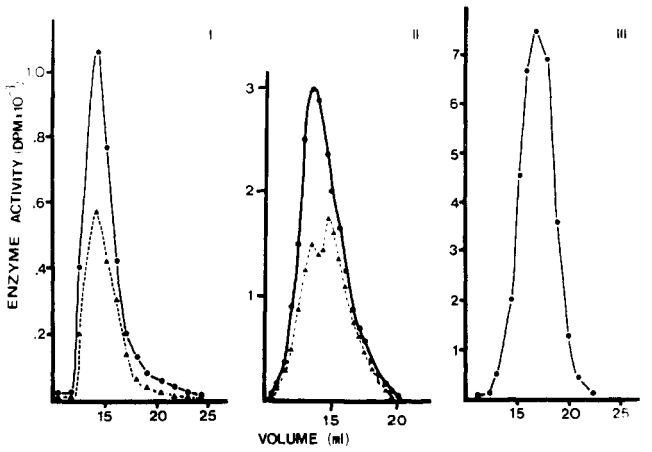

FIGURE 1: Elution profiles of three samples of orotate phosphoribosyltransferase and orotidylate decarboxylase, of various ages, on Sephadex G-75. The enzymes were eluted with 50 mM potassium phosphate, pH 7.4, at 4 °C. (I) A fresh sample of the 62 000 molecular weight form of the enzyme complex with phosphoribosyltransferase specific activity equal to one half that of decarboxylase specific activity when assayed with substrate alone. Both enzymes eluted at the same volume: decarboxylase (●-●), phosphoribosyltransferase (▲- - -▲). (II) A sample of the 62 000 molecular weight form of the enzyme complex which had been stored at −20 °C for 2 months. Only 30% of the phosphoribosyltransferase was measurable by substrate alone. (Results were obtained in the absence of dithiothreitol. In the presence of dithiothreitol the total phosphoribosyltransferase activity was one half of the decarboxylase activity.) The decarboxylase activity (●-●) eluted in a single peak, while there appeared to be a double peak of phosphoribosyltransferase (▲- - -▲) activity. (III) A sample of decarboxylase which had been stored at −20 °C for >6 months. No phosphoribosyltransferase activity was detectable either with substrate alone or with dithiothreitol in the assay mixture. The decarboxylase eluted in a single peak at a volume corresponding to a molecular weight of ~35 000. (Samples of 0.5 mL were collected in all cases but only every second tube is indicated, except for II.)

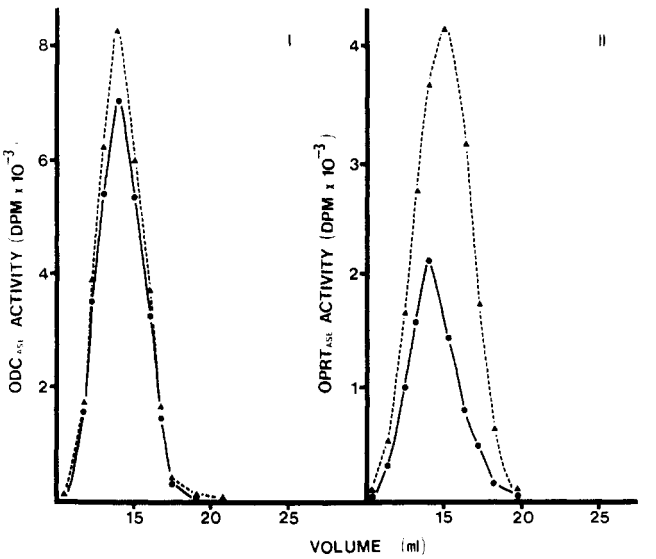

FIGURE 2: An aliquot of the enzyme used in the experiment described in Figure 1(II) was applied to the Sephadex G-75 column and eluted with 50 mM potassium phosphate, pH 7.4, at 4 °C as before. Each fraction was divided into four equal parts and these were assayed for orotidylate decarboxylase and orotate phosphoribosyltransferase activities with and without 1 mM dithiothreitol and 10 μM EDTA in the reaction mixture. Figure 2 (I) shows the elution profile for decarboxylase: with dithiothreitol (▲- - -▲), and without dithiothreitol (●-●). There is a single peak with the same elution volume. Figure 2(II) shows the results for phosphoribosyltransferase: with dithiothreitol (▲- - -▲), and without dithiothreitol (●-●); in this case, the peaks correspond to molecular weight species of approximately 50 000 and 62 000, respectively.

presented in Figure 1(II) are further clarified in Figure 2. The same enzyme was run on the same column but the fractions were divided into four equal volumes. These were assayed for both activities in the presence and absence of dithiothreitol. The decarboxylase eluted as a single peak under both conditions with the same elution volume. The phosphoribosyltransferase eluted in two peaks with different elution volumes corresponding to a molecular weight difference of ~12 000 (i.e., ca. 62 000 and 50 000, respectively).

(*B*) *Effect of Incubation with Guanidine Hydrochloride on Specific Activity.* The effect of incubation in a range of guandine hydrochloride concentrations and for different time periods on the activity of orotidylate decarboxylase (preparation I) is shown in Figure 3. Essentially similar experiments were carried out with the phosphoribosyltransferase (specific activity, 90 nmol mg^{-1} h^{-1}) but with guanidine hydrochloride concentrations from 0.05 to 0.28 M; i.e., concentrations of guandine hydrochloride were chosen to give approximately the same range of $T_{1/2}$, the time for 50% inactivation, for the two enzymes, respectively.

Under the experimental conditions, the inactivation of both enzymes was essentially irreversible, as finite small concentrations of guanidine hydrochloride remained when the sample was diluted up to the final assay volume. Though inactivation appeared to follow a first-order rate relationship for both enzymes, the rate of inactivation was related to the concentration of guanidine hydrochloride in a different manner for the two enzymes (Figure 4). For orotate phosphoribosyltransferase, the rate was linearly proportional to guanidine hydrochloride concentration in the range studied (0.05-0.28 M). For orotidylate decarboxylase, the inactivation only occurred at a comparable rate at higher guanidine hydrochloride concentrations (0.1-1.0 M) and there appeared to be an exponential relationship between the rate of inactivation and the concentration of the denaturing agent. Above 0.5 M, the rate of inactivation became approximately proportional to the guanidine hydrochloride concentration.

In Figure 5 the effect of including 1 mM dithiothreitol and 10 μM EDTA in the assay mixture into which the samples of enzyme were diluted after incubation in guanidine hydrochloride is demonstrated. These compounds had no effect on the level of activity of the orotidylate decarboxylase remaining after various periods of time in 0.3 M guanidine hydrochloride. There was, however, a dramatic effect on the orotate phosphoribosyltransferase activity. The first-order inactivation was preserved but the level of activity was raised considerably, viz., the initial activity was 2.5 times the activity assayed in the absence of dithiothreitol. At the end of the time period (160 min), the activity of enzyme assayed in the presence of the thiol reagent was still substantially greater than the initial control activity.

The effect of including dithiothreitol in the guanidine hydrochloride incubation mixture rather than in the diluting medium was also tested. There was little effect on the decarboxylase activity but again a marked effect on the phosphoribosyltransferase activity. The initial activity of the latter enzyme was again 2.5 times greater in the dithiothreitol-containing system than in the control. During incubation in the guanidine hydrochloride–dithiothreitol mixture, the specific activity of the orotate phosphoribosyltransferase increased still further in contrast to the results shown in Figure 5; at the end of the 2-h incubation, its activity in the guanidine hydrochloride–dithiothreitol mixture was five times the initial control value and eight times the final control value.

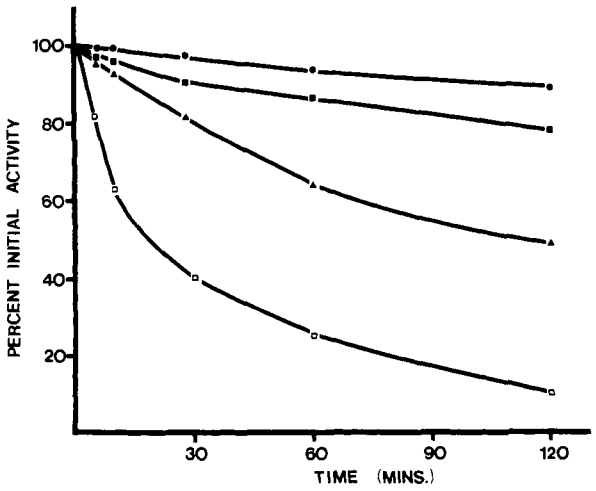

FIGURE 3: Time course of inactivation of orotidylate decarboxylase (preparation I) by guanidine hydrochloride at various concentrations. Enzyme activity is expressed as a percent of the control which was treated in the same manner as the test solutions without addition of guanidine hydrochloride. A sample of enzyme (specific activity 220 nmol mg^{-1} h^{-1}) was incubated at 4 °C in the presence of guanidine hydrochloride for the times shown, when an aliquot was removed and diluted into the final assay mixture. The diluted enzyme was kept at 4 °C until all aliquots were taken, at which stage the samples were brought to 37 °C and the assay was initiated by the addition of [*carboxyl*-^{14}C]OMP. Results are shown for four guanidine hydrochloride concentrations, 0.1 M (●-●), 0.25 M (■-■), 0.5 M (▲-▲), and 1.0 M (□-□). The concentration of guanidine hydrochloride in the final assay mixture was 0.05 M and the protein concentration was 0.5 mg/mL.

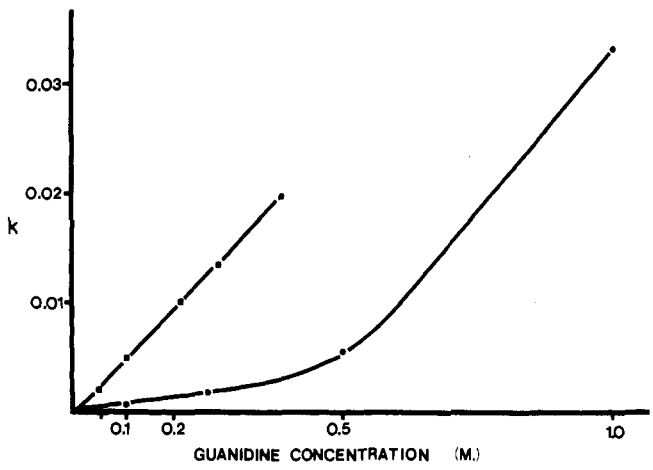

FIGURE 4: Inactivation of orotate phosphoribosyltransferase (■-■) and orotidylate decarboxylase (●-●) activities as a function of guanidine hydrochloride concentration. The data from the inactivation experiments (e.g., Figure 3 for the decarboxylase) were used to calculate k, the inactivation rate constant for a particular guanidine hydrochloride concentration, using the expression $N_t = N_0 e^{-kt}$, where N_t is the fraction of activity remaining at time t and N_0 the initial activity.

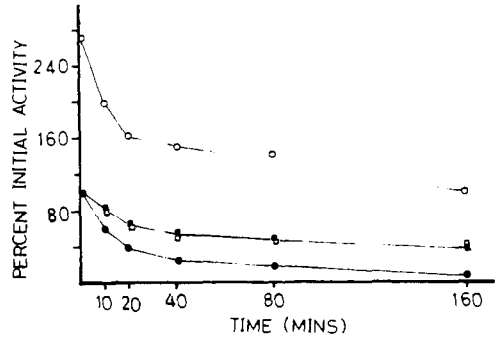

FIGURE 5: Time course of inactivation of orotidylate decarboxylase and orotate phosphoribosyltransferase by guanidine hydrochloride at a concentration of 0.3 M. In this experiment, two aliquots were withdrawn for each enzyme at the indicated time intervals. One was diluted into the standard reaction mixture and the other into a reaction mixture which contained 1 mM dithiothreitol and 10 μM EDTA. Results are expressed as a percent of the control as described in the legend to Figure 3. Four situations are described: decarboxylase diluted into the standard assay mixture (■-■) and into dithiothreitol and EDTA (□-□), and phosphoribosyltransferase diluted into its normal reaction mixture (●-●) and into dithiothreitol and EDTA (○-○). The final guanidine hydrochloride concentration was 0.05 M and protein concentration was 0.5 mg/mL. The enzyme sample used was the same as in Figure 3.

In separate experiments, the inactivation of orotidylate decarboxylase at low protein concentration was demonstrated to follow first-order kinetics as observed in this laboratory and by other workers (Brown et al., 1975; Smith et al., 1961; Krooth et al., 1973). Further, the sulfhydryl group reagent, *p*-chloromercuribenzoate, at a concentration of 50 μM, totally abolished orotate phosphoribosyltransferase activity after 30 min at 37 °C but had almost no effect on orotidylate decarboxylase under the same conditions.

(*C*) *Effect of Incubation with Guanidine Hydrochloride on Molecular Weight.* The disaggregation effects of guanidine hydrochloride on the 62 000 molecular weight complex (preparation 1) was studied in two ways. Firstly, samples of the enzyme were incubated with 0.2 M guanidine hydrochloride for 1 h at 4 °C and then applied to a Sephadex G-75 column and eluted with 50 mM potassium phosphate, pH 7.4. Aliquots of the fractions were taken for assay of both enzymes and the remainder of each fraction was retained for recombination experiments. In the second series of experiments, the enzyme was applied to the Sephadex G-75 column and eluted with 50 mM potassium phosphate, pH 7.4, containing various concentrations of guanidine hydrochloride. The fractions were treated as above.

Results from these two types of experiments were similar and typical patterns are presented in Figure 6. A peak containing both enzyme activities rechromatographed at the 62 000 molecular weight position, the recovery depending on the concentration of guanidine hydrochloride used. For example, when the enzyme was incubated for 1 h at 4 °C in 0.2 M guanidine hydrochloride (in the absence of dithiothreitol), before applying it to the column, recovery of the decarboxylase was 45% and recovery of the transferase was 15%. However, on further elution, different profiles were obtained for the two enzymes. Firstly, a second peak of orotidylate decarboxylase, which represented ~30% of the total activity recovered, eluted at a position corresponding to molecular weight ~20 000. Secondly, in addition to the major peak of activity, two further peaks of recoverable orotate phosphoribosyltransferase activity could be detected. These peaks, as illustrated by the dotted line in Figure 6, had elution volumes which corresponded to molecular weights of approximately 25 000 and 13 000, respectively. (Detection of the activity in the assay system implies that the guanidine hydrochloride was sufficiently diluted out (four to tenfold) so that recombination of the phosphoribosyltransferase subunits with the decarboxylase could occur.) The peaks of activity were approximately equal and accounted for a total of 30% of the recovered activity of the phosphoribosyltransferase. Details of recombination experiments used to further characterize these peaks are given in the next section.

The existence of these different forms of the enzymes and assignment of molecular weight values to them are based on

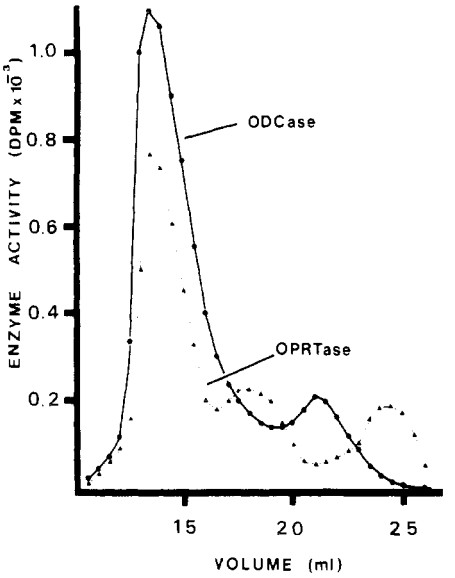

FIGURE 6: Chromatography of a sample of orotate phosphoribosyltransferase-orotidylate decarboxylase, in the 62 000 molecular weight form, on Sephadex G-75 (column size 0.8 × 40 cm). The enzyme was preincubated for 1 h at 4 °C in 0.2 M guanidine hydrochloride before being applied to the column. Elution of the enzymes was with 50 mM potassium phosphate, pH 7.4, at 4 °C. Fractions of 0.5 mL were collected and 200-μL aliquots of each fraction were assayed for both enzyme activities in the standard assay mixtures. Protein concentration in the assay system was brought to 0.5 mg/mL where necessary by addition of human serum albumin. Recovery of the decarboxylase activity (●-●) was 45%, and recovery of the phosphoribosyltransferase (▲- - -▲) was 15%.

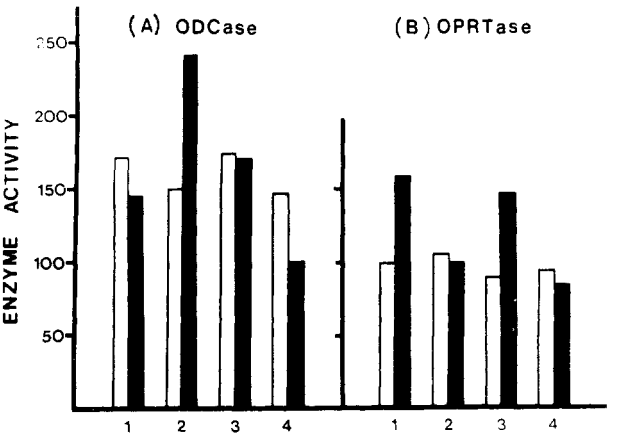

FIGURE 7: Recovery of activity on recombination of subunits. Fractions obtained from the Sephadex G-75 column described in Figure 6 were pooled as follows. Four pairs of samples were taken with the following elution volumes: (1) 17-19 mL, (2) 20-22 mL, (3) 23-25 mL, and (4) 26-28 mL. The remaining volumes of fractions eluting between 12 and 16 mL were also pooled. One half of each of the samples 1-4 was mixed with an equal volume of 50 mM potassium phosphate, pH 7.4, containing 2 mg/mL human serum albumin. The other half of each sample was mixed with an equal volume of the pooled fractions eluting between 12 and 16 mL. An aliquot of this latter pooled sample was also mixed with an equal volume of phosphate buffer and human serum albumin. All samples were dialyzed for 4 h against two changes of 50 mM potassium phosphate, pH 7.4, at 4 °C. They were brought to 37 °C, divided into two equal volumes, and assayed for orotidylate decarboxylase and orotate phosphoribosyltransferase by addition of the relevant substrates. Figure 7A shows the results for decarboxylase and Figure 7B the results for phosphoribosyltransferase. The open columns are the sum of the activities of the two components when dialyzed separately (in the presence of albumin) against phosphate buffer. The adjacent solid column is the activity resulting when the two components were dialyzed together.

the assumption that no equilibrium effects are operating during the gel-filtration experiments (Nichol et al., 1964). Though this assumption cannot be directly supported in the present study, the results are most simply explained on such a basis. We would note that the elution profile was the same when dissociation with guanidine hydrochloride was carried out before or during chromatography. Further, previous studies on the dissociation of the enzyme at low protein concentration (Brown et al., 1975) are consistent with such an assumption.

Recovery of Activity on Recombination of Subunits. The fractions from the column chromatography experiments illustrated in Figure 6 were pooled, dialyzed, and reassayed in various combinations as described in the legend to Figure 7. The activities of a combination of each of the four pooled fractions with an aliquot of the basic 62 000 molecular weight form are compared with the sum of the activities of the two components dialyzed separately. The combination of the basic complex and enzyme eluting between 20 and 22 mL (corresponding to the small peak of decarboxylase activity in Figure 6) was the only one which showed greater activity than the sum of the two components treated separately. Similarly, recoverable activity from the lower molecular weight fractions of the phosphoribosyltransferase was only detected at positions corresponding to the two small peaks identified by the initial Sephadex G-75 chromatography. In other words, phosphoribosyltransferase activity could be recovered by the addition of protein eluting at positions corresponding to molecular weights of 25 000 and 13 000, respectively, to solutions containing decarboxylase activity.

Kinetic Properties of Various Forms of Orotate Phosphoribosyltransferase and Orotidylate Decarboxylase. Some basic kinetic properties of the two enzymes in their various forms were determined. Different kinetic parameters were obtained for orotate phosphoribosyltransferase in the presence and absence of dithiothreitol. The enzyme sample used had 30% of the total activity measurable with substrates alone and the remainder requiring the presence of dithiothreitol. In the presence of the latter compound, with PRibPP, 250 μM, and orotic acid between 5 and 220 μM, both the K_M and the V_{max} values were approximately fourfold greater than in its absence: viz., K_M values of 10 and 36 μM and V_{max} values of 32 and 144 nmol mg^{-1} h^{-1}, in the absence and presence of dithiothreitol (1 mM), respectively.

Kinetic experiments were also carried out on the 35 000 molecular weight form of decarboxylase. This form had no detectable phosphoribosyltransferase activity. The double-reciprocal plot was found to be bimodal, concave upwards, and demonstrated two K_M values, of 200 and 12 μM, at low and high OMP concentrations, respectively. The apparent activation of the enzyme by increasing OMP concentration was further studied by examining the effect of OMP on the molecular weight of this form of decarboxylase. An aliquot of the enzyme was incubated for 90 min at 37 °C in the presence of 1 mM OMP. It was then applied to a Sephadex G-75 column and eluted with 50 mM potassium phosphate, pH 7.4. There was significant conversion to a form of the enzyme of molecular weight ~80 000 under these conditions. Incubation under the same conditions with buffer alone, or with an equivalent amount of UMP, did not result in any aggregation of the 35 000 molecular weight form of decarboxylase.

Discussion

A number of observations have indicated a subunit structure for the 62 000 molecular weight form of the orotate phos-

phoribosyltransferase-orotidylate decarboxylase from human erythrocytes. Evidence for the presence of subunits was obtained from analysis of the rate of inactivation of both enzymes in guanidine hydrochloride, combined with gel filtration studies of the subunits formed during storage and following preincubation in guanidine hydrochloride. The results suggest that the two catalytic activities are associated with, or possibly, reside on, different polypeptide chains, which can be distinguished by differences in their molecular weight. In the form isolated, the separate subunits do not realize their full activity, although a substantial amount of the original activity can be restored by recombination of the subunits.

Information on the subunit structure of the basic 62 000 molecular weight form of the enzyme complex may be summarized as follows.

(1) In some aged preparations, a molecular weight decrease of approximately 13 000 is observed (Figure 1(II)) to yield a 50 000 molecular weight species. This is associated with apparent loss of phosphoribosyltransferase activity, which can, however, be restored by the addition of dithiothreitol. No loss of decarboxylase activity appears to have occurred with this species.

(2) The rate of inactivation of the two enzyme activities by guanidine hydrochloride as a function of concentration of the denaturing agent is different for the two enzyme activities.

(3) Prolonged aging, or treatment with guanidine hydrochloride, resulted in complete loss of phosphoribosyltransferase activity but substantial retention of decarboxylase activity associated with a 35 000 molecular weight species.

(4) Protein eluting from Sephadex G-75 at positions corresponding to molecular weights of 25 000 and 13 000 manifested phosphoribosyltransferase activity when added to solutions containing decarboxylase activity (Figures 6 and 7).

The most intriguing subunit is that of molecular weight 50 000 which retains full decarboxylase activity but requires a thiol agent to express phosphoribosyltransferase activity. It would appear that the thiol agent not only retards or prevents the second step of the dissociation sequence but results in an apparently increased activity due to the higher V_{max} of the enzyme under those conditions (see Results). Thus, the activity is markedly increased in the presence of dithiothreitol but abolished by *p*-chloromercuribenzoate. Further treatment with guanidine hydrochloride, or aging, results in a 35 000 molecular weight species which has full decarboxylase activity but no restorable phosphoribosyltransferase activity. The simplest explanation for these observations is that the change in molecular weight from 62 000 to 35 000, via a 50 000 species, represents the sequential loss of two subunits that are essential for phosphoribosyltransferase activity. Further, the 35 000 molecular weight species appears to contain two subunits, which are presumed to be inactive in the monomer (~20 000) form, though activity is restored, presumably due to reaggregation, on dilution of guanidine hydrochloride and the addition of the substrate, OMP, as occurs in the reaction mixture.

A model for the basic 62 000 molecular weight unit may be proposed with the following features.

(a) Two subunits (~13 000 mol wt) responsible for phosphoribosyltransferase activity attached to two decarboxylase subunits (~20 000 mol wt) constitute the 62 000 molecular weight unit of the complex.

(b) The decarboxylase subunits are essentially inactive in the monomer (~20 000 mol wt) form; activity requires the formation of at least the dimer (~35 000 mol wt).

(c) The manifestation of phosphoribosyltransferase activity requires the attachment of the smaller (13 000 mol wt) subunits to at least two decarboxylase subunits.

(d) Removal of one of the 13 000 molecular weight subunits results in complete loss of phosphoribosyltransferase activity, unless the enzyme is assayed in the presence of dithiothreitol.

Somewhat similar results have been obtained from this laboratory in studies of both enzyme activities in extracts from human liver. The decarboxylase was found to exist in a number of molecular weight forms, the smallest unit detected having a molecular weight of 35 000. Phosphoribosyltransferase activity was not associated with this form, though it was detected in association with the decarboxylase in a species with a molecular weight of approximately 63 000 (Campbell et al., 1977). Kasbekar et al. (1964) have claimed separation of the two enzyme activities by starch gel electrophoresis of the partially purified enzymes from calf thymus. Reyes and Guganig (1975) also obtained an apparent separation of the two enzyme activities on a sucrose gradient following treatment of the phosphoribosyltransferase-decarboxylase complex from murine leukemia P1534J with elastase.[2]

The results showing aggregation of the 35 000 molecular weight form of the erythrocyte decarboxylase during incubation with OMP suggest that decarboxylase subunits separated from phosphoribosyltransferase subunits can still behave in an analogous fashion to the complete complex (Brown et al., 1975). The activation of the enzyme by its substrate is of particular interest. The K_M decreases to a value comparable to that obtained for the basic complex when the OMP concentration increases and this is associated with dimerization of the enzyme. Previous studies of the complete complex showed that the K_M for OMP also progressively decreased with increasing aggregation (Brown et al., 1975). We could note that other workers have also observed different K_M values for the decarboxylase from various sources, although this has not always been associated with changes in aggregation state of the enzyme (e.g., Appel, 1968; Fyfe et al., 1973).

The results presented here account for the copurification of orotate phosphoribosyltransferase and orotidylate decarboxylase and also the greater lability of the phosphoribosyltransferase as previously described for the enzymes from mammalian tissues (Kasbekar et al., 1964; Appel, 1968; Brown et al., 1975; Grobner and Kelley, 1975). The difficulty in separating the two enzyme activities can be compared to the situation in yeast, where the two enzymes are readily separable (Umezu et al., 1971). Also, the yeast decarboxylase is particularly sensitive to sulfhydryl reagents and the phosphoribosyltransferase is insensitive (Umezu et al., 1971; Creasey and Handschumacher, 1961). The opposite situation applies for the enzymes isolated from human erythrocytes.

The model suggested for the 62 000 molecular weight form of the phosphoribosyltransferase-decarboxylase complex could provide an explanation of the defect of both enzymes in hereditary orotic aciduria and the selective defect in the variant

[2] The possibility that our results could be attributed to limited proteolysis of a single polypeptide chain, as has been observed, for example, for eucaryotic fatty acid synthetase (Stoops et al., 1975), appears remote. The exhaustive washing procedures (see Materials and Methods) would have removed any white cells present. Similar results were obtained for different preparations of different degrees of purity and the results of aging experiments were similar to those obtained with guanidine hydrochloride. Further, evidence has been obtained for a 35 000 molecular weight form of the decarboxylase from human liver, in the presence of a protease inhibitor (soybean trypsin inhibitor) and also for a ~40 000 molecular weight form from human spleen (L. Kelly and W. J. O'Sullivan, unpublished results). In neither instance was there any associated phosphoribosyltransferase activity.

forms of the disease based on a structural defect in one or other of the enzyme subunits. Thus, a structural defect of the decarboxylase subunit abolishing formation of the active decarboxylase dimer would secondarily cause a defect in the phosphoribosyltransferase as subunits of this enzyme would remain unbound and hence inactive.

In the presence of potent inhibitors of decarboxylase, such as 6-azaUMP as in the work of Pinsky and Krooth (1967), sufficient conformational change in the decarboxylase subunit might occur to allow the active dimer form to exist. This would then bind phosphoribosyltransferase subunits and the complex could become fully active. The fact that this effect is only demonstrable in cells capable of synthesizing protein suggests that the subunits are very labile once formed; in fact, the phosphoribosyltransferase-decarboxylase subunits could be assembled as a complex during synthesis.

In the variant forms of hereditary orotic aciduria, structural defects involving the catalytic activity of the different subunits rather than affecting their ability to form the basic complex would be postulated. Recent results by Worthy et al. (1974) suggest that the enzymes from one patient with hereditary orotic aciduria do differ in properties from the enzymes obtained from normal subjects. The differences were, in particular, increased lability and altered migration on polyacrylamide gel electrophoresis. The latter was attributable to charge differences rather than different molecular weight forms. In addition, the kinetic properties of the mutant enzyme were identical to those of the normal enzyme. Further studies on the mutant forms of phosphoribosyltransferase and decarboxylase in patients with hereditary orotic aciduria will be necessary before a defect in a structural gene rather than in a regulator gene can be definitely implicated in this disease.

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