Purification and Properties of an Enzyme Duet, Phosphoribosylaminoimidazole Carboxylase and Phosphoribosylaminoimidazolesuccinocarboxamide Synthetase, Involved in the Biosynthesis of Purine Nucleotides *de novo*

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Purification and some properties of an adjacent pair of enzymes in the biosynthetic pathway to AMP, phosphoribosylaminoimidazole carboxylase (EC 4.1.1.21) and phosphoribosylaminoimidazolesuccinocarboxamide synthetase (EC 6.3.2.6), are described. The enzymes form a duet, which may be regarded as a single molecule with a dual function, or as two very similar substances.

The sequence of reactions leading to the biosynthesis of purine nucleotides *de novo* is well established (Buchanan & Hartman, 1959) and may (to AMP) be represented in schematic form: 5-phosphoribosyl α -D-pyrophosphate $\xrightarrow{E_1}$ 5-phospho- β -D-ribosylamine $\xrightarrow{E_2}$ N-glycyl-5-phospho- β -D-ribosylamine $\xrightarrow{E_3}$ N-(N-formylglycyl)-5-phospho- β -D-ribosylamine $\xrightarrow{E_4}$ 2-formamido-N- β -D-ribosylacetamidine 5'-phosphate $\xrightarrow{E_5}$ 5-amino-1- β -D-ribosylimidazole 5'-phosphate $\xrightarrow{E_6}$ 5-amino-1- β -D-ribosylimidazole-4-carboxylic acid 5'-phosphate $\xrightarrow{E_7}$ 5-amino-1- β -D-ribosylimidazole-4-N-carbonyl-L-aspartate 5'-phosphate $\xrightarrow{E_8}$ 5-amino-1- β -D-ribosylimidazole-4-carboxamide 5'-phosphate $\xrightarrow{E_9}$ 5-formylamino-1- β -D-ribosylimidazole-4-carboxamide 5'-phosphate $\xrightarrow{E_{10}}$ IMP $\xrightarrow{E_{11}}$ 6-N-succinoadenosine 5'-phosphate $\xrightarrow{E_{12}}$ AMP comprising 12 steps with 12 associated enzymes and feedback inhibition of E_1 with completed nucleotides.

The universality of the pathway, the control it exerts over cell growth and its presumed presence in the first primordial cell makes it of special philosophical importance. However, there has been relatively little published work on the purification, structure and properties of the various enzymes involved. Enzymes E_1 (amidophosphoribosyltransferase) (EC 2.4.2.14) and E_4 (phosphoribosylformylglycine amidinesynthetase) (EC 6.3.5.3) from avian liver have been purified by Rowe & Wyngaarden (1968) and Mizobuchi & Buchanan (1968) respectively. Enzyme E_1 was shown to have a molecular weight of 200 000, to contain non-haem iron and to readily dissociate into smaller subunits of molecular weight 100 000 or in the presence of thiols into units of molecular weight 50 000 (regarded as the monomer units), which were identical electrophoretically. Enzyme E_4 is recorded to have a molecular weight of 133 000 and also contains a reactive essential thiol group, which appears

to be involved in a polymerization process that can be inhibited by mercaptoethanol or dithiothreitol. Other special features associated with the pathway enzymes include (a) the identity of enzymes E_8 and E_{12} (adenylosuccinate lyase) (EC 4.3.2.2) (Flaks & Lukens, 1963) and (b) the suggestion that enzymes E_9 (phosphoribosylaminoimidazolecarboxamide formyltransferase) (EC 2.1.2.3) and E_{10} (IMP cyclohydrolase) (EC 3.5.4.10) are either the same enzyme with a dual function or two very similar molecular species that have so far resisted separation (Flaks *et al.*, 1957).

We now report results of experiments with the enzymes E_6 (phosphoribosylaminoimidazole carboxylase) (EC 4.1.1.21) and E_7 (phosphoribosylaminoimidazolesuccinocarboxamide synthetase) (EC 6.3.2.6).

Experimental

All operations were carried out at 5°C except where otherwise stated, and centrifugations were at 22000g for 20 min.

(a) *Enzyme purification.* The enzymes were extracted for 1 h from an acetone-dried powder (100 g) of fresh chicken liver, by 1 litre of 50 mM-Tris chloride containing 50 mM-NaCl and 6 mM-mercaptoethanol. The extract was centrifuged, the supernatant adjusted to pH 5.5 with 2 M-HCl, stirred for 10 min, and then centrifuged. The supernatant was adjusted to pH 8.0 with 2 M-NaOH and to it was slowly added cold (–30°C) acetone to 23% (v/v) concentration. The precipitate was removed by centrifugation and cold acetone was added to the supernatant to 35% concentration. The precipitate was collected by centrifugation and dissolved in the extraction buffer (200 ml). To this was added satd. $(\text{NH}_4)_2\text{SO}_4$ solution to 30% saturation and a precipitate removed as described above. More satd. $(\text{NH}_4)_2\text{SO}_4$ solution was added to 60% saturation, then the precipitate was collected by centrifugation and dissolved in the smallest amount of extraction buffer (approx. 80 ml.)

Fig. 1. Column chromatography of enzymes E_6 and E_7 (a) on CM-Sephadex and (b) on Sepharose 6B

(a) The eluting buffer was 10mM-Tris chloride, pH7.0, containing 6mM-2-mercaptoethanol, followed by a linear salt gradient (0–0.8M-NaCl) in the same buffer (see under 'Experimental' in the text); column size, 2.5cm $\times$ 40cm. (b) The eluting buffer was 10mM-Tris chloride, pH7.9 (see under 'Experimental' in the text); column size, 2.5cm $\times$ 95cm. —, E_{280} ; ●—●, enzyme E_6 activity; ○ · · · ○, enzyme E_7 activity. The units of enzyme activity are: E_6 , μ mol of 5-amino-1- β -D-riboseylimidazole-4-carboxylic acid 5'-phosphate/min per 10 μ g; E_7 , μ mol of 5-amino-1- β -D-riboseylimidazole-4-N-carbonyl-L-aspartate 5'-phosphate/min per 50 μ g.

The solution was desalted on a Sephadex G-25 column (2.5cm $\times$ 95cm) equilibrated with 10mM-Tris chloride, pH7.0, containing 6mM-mercaptoethanol, then applied to a CM-Sephadex column (2.5cm $\times$ 40cm) equilibrated with the same buffer, then eluted with a salt gradient derived from starting buffer (200ml) and starting buffer containing 80mM-NaCl (200ml) (Fig. 1a) and finally purified on a column (2.5cm $\times$ 95cm) of Sepharose 6B by elution with 10mM-Tris chloride, pH7.9; this removed a red protein (Fig. 1b).

(b) *Enzyme assay.* Enzyme E_6 was assayed either by measuring the increase in E_{250} it produced in a solution of 5-amino-1- β -D-riboseylimidazole 5'-phosphate

in 0.1M-Tris chloride containing 0.2M-KHCO₃ at pH7.9 or by the decrease in E of a solution of 5-amino-1- β -D-riboseylimidazole 4-carboxylic acid 5'-phosphate in 0.1M-Tris chloride at pH8.0. Enzyme E_7 was assayed by a new method in which 5-amino-1- β -D-riboseylimidazole-4-carboxylic acid 5'-phosphate (0.2mg) in 0.1M-Tris chloride is converted into 5-amino-1- β -D-riboseylimidazole-4-N-carbonyl-L-aspartate by incubation at 37°C for 10min in the presence of the enzyme, 30mM-MgCl₂, 30mM-L-aspartate, 36mM-ATP and KHCO₃ (20mg) in a total of 1.1ml. Unchanged intermediates, which would otherwise interfere with the subsequent assay, were removed by hydrolysis to acyclic products (Shaw, 1972) with a mixture of 1.33M-KH₂PO₄-H₃PO₄ (pH1.4), trichloroacetic acid (30%, w/v) and 2.5M-H₂SO₄ at 100°C (water bath) for 10min and the nucleotide was assayed directly by the Bratton-Marshall method (Bratton *et al.*, 1939).

Results

Throughout the whole of the extraction and purification procedures the two enzymes remained firmly together, with no evidence of any separation. The final material obtained appears to be pure and contains both enzymes E_6 and E_7 , which were also not further separated by isoelectrofocusing techniques.

The purity of the enzyme duet was further confirmed by gel electrophoresis in the presence of sodium dodecyl sulphate (Shapiro *et al.*, 1967), when only one major band with a molecular weight of about 52000 resulted (Plate 1a). When the freshly prepared enzyme duet was left at 4°C loss of activity (of each enzyme) occurred and electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulphate then showed two bands, one corresponding to a molecular weight of approx. 52000 and the other at a position corresponding to a molecular weight of about twice this value [(vi) and (vii) in Plate 1b]. If, however, the samples are incubated in mercaptoethanol as well as sodium dodecyl sulphate before electrophoresis the higher-molecular-weight band does not appear (Plate 1c). This suggests oxidation to an inactive dimer. Molecular-weight determinations of the enzyme duet on Sephadex G-200 indicated a value for molecular weight of about 350000 ($\pm$ 50000), suggesting that polymerization of a monomer unit of molecular weight 50000 occurs, and this is reminiscent of other enzymes so far examined in the pathway. In addition, the activity of each enzyme in the duet was similarly and simultaneously affected by various operations, including heat and also by *p*-chloromercuribenzoate (Boyer, 1954), suggesting a thiol involvement in each mechanism. On the other hand, iodoacetamide failed to affect either the thiol titre (indicating about 4.7 SH groups/unit of molecular

EXPLANATION OF PLATE I

Electrophoresis of enzymes E₆ and E₇ and standards on polyacrylamide gels

(a) Electrophoresis was carried out in 6% (w/v) polyacrylamide disc gels in 0.1% sodium dodecyl sulphate at 5 mA/tube for 5.25 h. (i), Apoferritin; (ii), γ -globulin; (iii), serum albumin; (iv), ovalbumin; (v), myoglobin; (vi), E₆-E₇ duet after CM-Sephadex chromatography; (vii), E₆-E₇ duet after further chromatography on Sepharose 6B. Samples were incubated in 10 mM-sodium phosphate buffer, pH 7.1, containing sodium dodecyl sulphate (1%, w/v) for 10 min at 100°C before electrophoresis. (b) Electrophoresis was carried out in 5% (w/v) polyacrylamide disc gels in 0.1% sodium dodecyl sulphate at 4 mA/tube for 16 h. (i), γ -Globulin; (ii), serum albumin; (iii), ovalbumin; (iv), myoglobin; (v), pepsin; (vi), E₆-E₇ duet fraction after chromatography on CM-Sephadex followed by Sepharose 6B; (vii), as for (vi) but with twice the sample size. Samples were prepared as indicated in (a). (c) Electrophoresis was carried out as described under (b) except that the incubation solvent system included 2-mercaptoethanol (1%, w/v).

weight 50000) or the activity of either enzyme, but in the presence of urea, iodoacetamide decreased the number of SH groups from 4.7 to 1.5/unit. On the other hand, the pH optima of enzymes E_6 (6.5) and E_7 (8.0) differed. *N*-Terminal analysis by the dansylation procedure (Hartley, 1970) showed the presence of two end groups, namely glycine and alanine.

Previous workers (Ahmad *et al.*, 1965), using impure enzyme preparations, have postulated a Mg^{2+} requirement for enzyme E_6 . Our results have shown that Mg^{2+} (to 33 mM) did not increase the rate of the forward reaction (5-amino-1- β -D-ribosylimidazole 5'-phosphate $\rightarrow$ 5-amino-1- β -D-ribosylimidazole-4-carboxylic acid 5'-phosphate) but did increase the extent of the reaction. Ni^{2+} was shown to have a similar effect at a much lower concentration and this was abolished by EDTA. Litchfield & Shaw (1971) have already shown that Ni^{2+} readily forms a stable complex with 5-amino-1- β -D-ribosylimidazole-4-carboxylic acid 5'-phosphate that alters the u.v.-absorption spectrum at pH 4.5, and Mg^{2+} was found to have a similar effect on the spectrum at pH 6.1. Since the maximum limits of impurities in the $MgSO_4$ used were 0.0005% the modified spectrum was probably a real effect due to Mg^{2+} , and this was confirmed by the fact that small amounts of EDTA had no effect on the Mg^{2+} -nucleotide spectrum. The results suggest that Mg^{2+} does not affect enzyme E_6 but modifies the extent of the reaction by complexing with the nucleotide and thereby decreasing its effective concentration.

Discussion

Our results indicate that enzymes E_6 and E_7 comprise an enzyme duet, which may be one molecule

with a dual function, or alternatively two very similar molecules with a propensity to undergo some degree of additional polymerization from a single 50000 unit to one of about 350000 (± 50000). From our own and other published work it would appear that a meaningful pattern of structure and function is already appearing in the pathway in terms of subunit size, thiol involvement and possible dual nature (or extremely close structural relationship) of at least three pairs of the 12 enzymes involved.

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