Adenylosuccinase Activity in Human and Rabbit Erythrocyte Lysates

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SUMMARY

Adenylosuccinase activity was demonstrated in lysates prepared from mature human and rabbit erythrocytes by investigating both reactions catalyzed by the enzyme. The conversion of adenylosuccinate to adenosine 5'-phosphate was observed spectrophotometrically. The conversion of N-(5-amino-1-ribosyl-4-imidazolecarboxyl)-L-aspartic acid 5'-phosphate to 5-amino-1-ribosyl-4-imidazolecarboxamide 5'-phosphate was studied by measuring the incorporation of sodium ({}^{14}C)-formate into the hypoxanthine of inosine 5'-phosphate when lysates were incubated with N-(5-amino-1-ribosyl-4-imidazolecarboxyl)-L-aspartic acid 5'-phosphate.

The presence of adenylosuccinase in the erythrocytes of both species suggests that the human erythrocyte, which cannot effect significant conversion of inosine 5'-phosphate to adenosine 5'-phosphate, lacks adenylosuccinate synthetase activity, an enzymatic capacity possessed by the rabbit erythrocyte. The data also suggest that the inability of the erythrocytes of both species to synthesize the purine ring by the de novo pathway results from the loss of one or more steps prior to the formation of N-(5-amino-1-ribosyl-4-imidazolecarboxyl)-L-aspartic acid 5'-phosphate.

The inability of human erythrocytes to effect significant conversion of inosine 5'-phosphate to adenosine 5'-phosphate in vitro (1, 2), is in marked contrast to the ability of the rabbit erythrocyte to make this conversion (3, 4), in which adenylosuccinate is an intermediate (5). Although the human red blood cell is deficient in one or both of the two enzymes involved in the conversion, adenylosuccinate synthetase, or adenylosuccinase.

In addition to its role in the cleavage of adenylosuccinate, adenylosuccinase is also required for the conversion of N-(5-amino-1-ribosyl-4-imidazolecarbonyl)-L-aspartic acid 5'-phosphate to 5-amino-1-ribosyl-4-imidazolecarboxamide 5'-phosphate, a reaction of the de novo pathway prior to the completion of the purine ring. Although mature erythrocytes of both species can form IMP from AICAR, they are unable to synthesize purine nucleotides de novo (6). Because of the dual function of adenylosuccinase (7, 8), it was of interest to investigate its presence in erythrocyte lysates.

The availability of samples of adenylosuccinate and SAICAR, the latter prepared from an adenylosuccinase and adenylosuccinate synthetase-deficient yeast mutant, has made it possible to demonstrate the presence of adenylosuccinase in mature human and rabbit erythrocytes.

METHODS

Spectrophotometric Experiments—Human erythrocytes from normal adults were prepared from freshly drawn whole blood collected into a small volume of standard acid-citrate-dextrose or heparin solution. Rabbit blood, obtained from an ear vein or by cardiac puncture, was collected into heparin solution. Erythrocytes were washed three times by resuspension in isotonic (0.9%) NaCl solution. The buffy coat was removed.

Lysates were prepared by the addition of 1 volume of water to 2 volumes of packed cells, and freezing and thawing the suspension three times in a Dry Ice-alcohol bath. Stroma-free lysates were obtained by centrifugation of total cell lysates at 12,000 x g for 10 min at 4°C. Lysates were incubated alone or in the presence of either added purine nucleotides or sodium phosphate, or both, as indicated in the legends to the figures. All incubations were carried out at 37°C in a metabolic shaker for the times indicated in the legends.

Preparation and Assay of Extracts—Extracts were prepared by adding aliquots of the incubation suspension to at least 10 ml of assay mixture. The availability of samples of adenylosuccinate and SAICAR, the latter prepared from an adenylosuccinase and adenylosuccinate synthetase-deficient yeast mutant, has made it possible to demonstrate the presence of adenylosuccinase in mature human and rabbit erythrocytes.

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The abbreviations used are: AICAR, 5-amino-1-ribosyl-4-imidazolecarboxamide 5'-phosphate; SAICAR, N-(5-amino-1-ribosyl-4-imidazolecarbonyl)-L-aspartic acid 5'-phosphate; AICA, 5-amino-1-ribosyl-4-imidazolecarboxamide; PP-ribose-P, 5-phosphoribosyl-1-pyrophosphate; ribosyl AICA, 5-amino-1-ribosyl-4-imidazolecarboxamide; ribosyl SAICA, N-(5-amino-1-ribosyl-4-imidazolecarbonyl)-L-aspartic acid.
volumes of cold 2% perchloric acid. After centrifugation, the supernatant solutions were assayed spectrophotometrically by measuring their absorbance at 250, 260, 270, and 290 nm. The absorbance values of the control samples, to which purine nucleotides had not been added, were subtracted from the experimental samples, and optical density ratios were determined. Percentage deamination or percentage of IMP or of AMP was calculated on the basis of the 250/260, 270/260, or 280/260 absorbance ratios of each extract and their proportionality to the corresponding ratios of an extract containing a known single purine nucleotide.

Isotopic Experiments—Lysates, prepared by hypotonic lysis in 3 volumes of 0.005 M MgCl₂, were incubated with sodium (²⁵C) formate alone, or together with AICA plus PP-ribose-P, ribosyl AICAR, or SAICAR at 37° for 30 min. Perchloric acid extracts were prepared. The extracts were heated in a boiling water bath for 1 hour and silver salts of the purines were precipitated. The free purines were extracted from the silver salts with dilute HCl and were chromatographed on paper in several solvent systems. Individual spots eluted from the paper were assayed spectrophotometrically. The eluates were assayed for radioactivity in a Nuclear Chicago gas flow counter.

Preparation and Characterization of N-(5-Amino-1-ribozylo-(4-imidazolecarbonyl)-2-aspartic acid 5'-phosphate—SAICAR was collected as the accumulation product of a double mutant strain of Saccharomyces cerevisiae, A664a/10B, lacking adenylosuccinase, ade5, and adenylosuccinate synthetase, ade12 (9).

The cells were grown overnight in a rotary incubator-shaker to about 100-fold increase, at 30°, in 3 liters of a supplemented Difco yeast nitrogen base medium, GBHA (9), with glucose reduced to 10 g per liter. They were washed and resuspended in 3 liters of the same medium, lacking adenine, for 5 hours, before harvesting by centrifugation. The cell pellet, 8 ml, was extracted five times with 5% trichloracetic acid at room temperature. The total extract, 40 ml, was adjusted to pH 7.1 with 0.1 M NaOH. SAICAR was precipitated as the barium salt by the addition of 0.1 ml of 1 M barium chloride and 3 volumes of ethanol. The supernatant solution contained ribosyl SAICA. The barium pellet was extracted with five 1-ml portions of 0.01 M HCl and the barium was reprecipitated as barium sulfate.

The identification of SAICAR in the extract was based on the presence of a single chromatographic band at an Rf of 0.20 in n-butanol-glacial acetic acid-water (60:15:25), by ultraviolet absorption, and by positive Foulty (10) and Bratton-Marshall assays (11). Treatment of the SAICAR with alkaline phosphatase (Worthington, EC 3.1.3.1) resulted in a Bratton-Marshall-positive product that chromatographed at an Rf of 0.38, corresponding to ribosyl SAICA.

For use of the Bratton-Marshall assay as a chromatographic location reagent, the paper was sprayed with 10% sulfuric acid, 5% sodium nitrite, 0.5% ammonium sulfamate, and 0.1% N-(1-naphthyl)-ethylenediaminohydrochloride in rapid succession.

MATERIALS

The sample of adenylosuccinate was kindly provided by Dr. C. W. H. Partridge. AICA and ribosyl AICA were purchased from Calbiochem. PP-ribose-P was purchased from Pabst Laboratories. Sodium (²⁵C)-formate was purchased from Schwarz BioResearch.

RESULTS

The presence of an active adenylic acid deaminase in the mammalian erythrocyte is well known (12). It is, in fact, responsible for a key reaction in the irreversible loss of adenine nucleotides in the erythrocytes of stored blood. When AMP is added to total cell lysates or stroma-free lysates prepared from mature rabbit or human erythrocytes, extensive deamination of the AMP occurs, with the formation of IMP (Fig. 1). In the human lysates, under the conditions employed, quantitative conversion occurs in about 30 min. The similarity of data obtained with total cell lysates and stroma-free lysates suggests that the deaminase is either a soluble enzyme or it is readily released from the cell membrane under these mild conditions.

Despite the rapid and complete deamination of added AMP after a short incubation period, it is possible to demonstrate the transient existence of AMP when adenylosuccinate is added to total cell lysates prepared from rabbit erythrocytes. As seen in Fig. 2A, the added adenylosuccinate was converted quantitatively to AMP within 10 min. The AMP thus formed gradually disappears with a concomitant increase in IMP concentration. The finding that adenylosuccinate is converted extensively to AMP in rabbit lysates is not unexpected in view of earlier findings that the intact rabbit erythrocyte is capable of converting IMP to AMP (3). The immediate and extensive formation of AMP also suggests that any direct formation of IMP, by reversal of the adenylosuccinate synthetase reaction, is minimal.

The data of Fig. 2, B and C, demonstrate that added adenylosuccinate is also converted rapidly and extensively to AMP by total cell lysates and stroma-free lysates prepared from mature human erythrocytes. The resemblance to the data obtained with rabbit lysates is marked, and indicates that the human erythrocyte possesses adenylosuccinate activity. The failure of the intact human erythrocyte to effect significant conversion of IMP to AMP may thus be attributed to the absence or inactivity of the enzyme, adenylosuccinate synthetase, alone, rather than to the absence of both enzymes required for the sequential conversion of IMP to adenylosuccinate to AMP.
The conversion of adenylosuccinate to AMP and its subsequent deamination to IMP by lysates prepared from rabbit and human erythrocytes. Adenylosuccinate (0.31 μmole in 0.20 ml) was added to 0.30 ml of cell lysate. Extracts were prepared and assayed as described. Percentage AMP and IMP were calculated.

When adenylosuccinate was added to lysates prepared from either rabbit or human erythrocytes, in the presence of 55 μmoles of phosphate ion by lysates prepared from rabbit and human erythrocytes, adenylosuccinate (0.31 μmole in 0.20 ml) was added to 0.30 ml of cell lysate. Extracts were prepared and assayed as described. Percentage AMP was calculated.

Since the enzymatic cleavage of SAICAR yields AICAR, which can serve as a precursor of IMP in erythrocytes of both species, the formation of labeled hypoxanthine of IMP, during the incubation of cell lysates with SAICAR and sodium (14C)-formate, would be indicative of adenylosuccinase action on SAICAR.

SAICAR, isolated from the yeast double mutant, ade12-ade13, was therefore studied as a potential precursor of IMP in erythrocytes. Incubation of lysates with sodium (14C)-formate alone or together with AICA plus PP-ribose-P, ribosyl AICA, or SAICAR, resulted in the data presented in Table I. Although no appreciable label appeared in the hypoxanthine of IMP isolated from lysates of either species incubated with sodium (14C)-formate, alone, significant incorporation of formate into hypoxanthine occurred in the presence of each of the three receptors or potential receptors of carbon 2 of the purine ring. The reactions involved here are as follows:

\[
\begin{align*}
AICA + PP\text{-ribose-P} \quad & \rightarrow \quad 5\text{-formamido-1-ribosyl}\text{-4-imidazolecarboxamide} \rightarrow \text{IMP} \\
\text{SAICAR} \quad & \rightarrow \quad \text{AICAR} \\
\text{ribosyl AICA} + \text{ATP} \quad & \rightarrow \quad 5'\text{-phosphate}
\end{align*}
\]

Evidence for the reactions of AICA and its ribosyl derivative in intact mammalian erythrocytes has been presented previously (6). When ribosyl SAICAR was incubated with sodium (14C)-formate in a comparable experiment with intact or lysed rabbit and human erythrocytes, no appreciable label was detected in the hypoxanthine of IMP. This finding is in contrast to the results obtained with ribosyl AICA and suggests that phosphorylation of ribosyl SAICAR may not occur in the erythrocytes of these species.

From the current data, it is apparent that lysates prepared from human and rabbit erythrocytes are capable of converting SAICAR to IMP, presumably via AICAR, in conformity with...
the appropriate portion of the single known pathway for synthesis de novo of purine nucleotides. In view of the dual function of adenylosuccinase, the isotopic data support the evidence obtained by spectrophotometry for the presence of the enzyme in the lysates investigated.

**DISCUSSION**

The data presented here extend our knowledge of the enzymatic deficiencies acquired during the final phase of human erythrocyte maturation. The demonstration that the human erythrocyte can convert adenylosuccinate to AMP serves to localize the deficiency in the conversion of IMP to AMP to that of adenylosuccinate synthetase, an enzymatic deficiency that probably arises during the maturation of the human reticulocyte (13). The absence of this enzymatic activity, together with the absence of earlier steps of the pathway leading to the synthesis of the purine nucleotide by the de novo pathway limits the human erythrocyte to replenishment of its adenine nucleotide pool from the free base, adenine, or a derivative (1, 14). In contrast, the rabbit erythrocyte can effect extensive conversion of either hypoxanthine, inosine, or adenosine to AMP via IMP (4).

The finding that the human erythrocyte possesses adenylosuccinase activity and the direct evidence for the conversion of SAICAR to AICAR is pertinent to our understanding of the final reactions of the de novo purine biosynthetic pathway in this cell. Earlier work in this laboratory demonstrated that rabbit and human erythrocytes are unable to carry out the sequence of reactions leading to the de novo synthesis of purine nucleotides, although the red cells of both species can carry out the final reactions in which AICAR is converted to IMP (1, 6). Studies with rabbit erythrocytes established the conversion of IMP to AMP and the probability that red cells of that species were capable of converting SAICAR to AICAR. The data presented here establish the latter capacity of the rabbit erythrocyte and demonstrate that human erythrocytes are also capable of effecting the conversion. It would appear, therefore, that the loss in ability for de novo synthesis of purine nucleotides which accompanies the maturation of the reticulocyte (18) is most likely due to the loss of one or more enzymatic activities in the sequence from PP-ribose-P to SAICAR. A recent study has suggested that PP-ribose-P amidotransferase activity is absent from human erythrocytes (15). The localisation of the missing step or steps in both species and the mechanism by which the loss occurs are currently under investigation.

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