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Stimulation and partial stabilization of human histidyl-tRNA synthetase by hemoglobin

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Histidyl-tRNA synthetase, an enzyme against which antibodies are directed in some patients with polymyositis, has been purified 5000-fold from HeLa cells, but was extremely labile to dilution or on storage at −80°C. In order to facilitate study of the biochemical and immunological properties of the enzyme, a stabilizer was sought. Hemoglobin at 2 mg/ml was found to stimulate the enzyme and also partially preserved the activity of the enzyme stored at a low concentration (< 10 μg/ml). Hematin, but not the globin protein, could substitute for hemoglobin in stimulating the enzyme.

Histidyl-tRNA synthetase; Autoantigen; Hemoglobin stabilization; Hematin

1. INTRODUCTION

Histidyl-tRNA synthetase (EC 6.1.1.21), an enzyme in the family of aminoacyl-tRNA synthetases, has assumed immunological significance as an autoantigen in the idiopathic inflammatory myopathies (polymyositis and dermatomyositis) [1]. It has been purified from several sources [2–4], and recently we have developed a rapid method for obtaining the human enzyme in high yield (5000-fold purified) from HeLa cells for immunological studies [5]. We have found, however, as had been reported for the enzyme from other sources [3,4], that it is markedly labile to dilution or storage. We sought, therefore, a method to preserve enzymatic activity for further studies. Among various agents tested, hemoglobin was found to enhance enzyme catalysis and, furthermore, to preserve partially the enzymatic activity of the preparation during storage at −80°C.

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2. MATERIALS AND METHODS

Bovine red blood cell ubiquitin, human hemoglobin, equine hemin (type III), bovine hemin (type I), human globin, salmon protamine sulfate, horse heart cytochrome *c*, and bovine serum albumin were from Sigma (St. Louis, MO); phenylmethylsulfonyl fluoride (PMSF), 5′-adenosine triphosphate, calf liver tRNA and dithiothreitol (DTT) were from Boehringer Mannheim Biochemicals (Indianapolis, IN); L-[^{3}H]histidine (spec. act. 54.4 Ci/mmol) was from New England Nuclear (Boston, MA). Analytical paper discs (0.5 in) were from Schleicher and Schuell (Keene, NH) and 24-well microtitre plates were from Costar (Cambridge, MA). All other reagents were the best analytical grade available. Hematin was prepared by dissolving hemin in 0.1 N NaOH immediately before use. The final pH of the solution was adjusted to 8.0. All other compounds tested as stabilizers were dissolved in water. Histidyl-tRNA synthetase was prepared as described [5]. Protein was estimated by the BioRad protein assay procedure [6] using BSA as the standard. The enzyme activity was assayed using a modified method of Kalousek and Konigsberg [7] in a total volume of 25 μl at 37°C for 10 min in 12 mM Tris-HCl buffer (pH 7.5) with 10 mM $MgCl_2$, 5 mM 5′-ATP, 0.6 mM DTT, 125 μg tRNA, 5 μl L-[^{3}H]histidine and an appropriate amount of enzyme protein. Parallel control assays were run without the enzyme in the presence of all the other components. Specific stabilizers were added to the enzyme prior to the addition of all the assay mixture components. After incubation for 10 min the reaction was stopped by adding 5 μl of cold 0.25 N HCl. An aliquot from each assay (25 μl) was then spotted onto analytical paper and

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dried, and each pad was separately incubated in a well of a 24-well microtitre plate with 10% trichloroacetic acid at 4°C for 20 min. The pads were washed with triple-distilled water, dried at 60°C and placed in 5 ml scintillation fluid. A unit of activity is defined as mmol × 10^7 L-[^{3}H]histidine incorporated as trichloroacetic acid-insoluble material in 10 min.

3. RESULTS

Histidyl-tRNA synthetase was purified 5000-fold from HeLa cells and the enzyme preparation, although not homogeneous, was found to contain no other aminoacyl-tRNA synthetase activities nor other antigens recognized by a variety of autoimmune sera [5]. Because of the immunological importance of the enzyme as an antigen in polymyositis, we wished to pursue studies of the enriched enzyme. We noted, however, that during the course of dilution of the enzyme, its specific activity fell sharply at protein concentrations below 10 μg/ml, with an 80% drop in specific activity at 5 μg/ml (fig.1). Above 10 μg/ml the specific activity remained relatively constant.

We attempted to restore the enzymatic activity by adding several substances frequently used for the purpose. Glycerol, glucose, BSA, protamine, cytochrome *c* and human globin, each added to a final concentration of 1 mg/ml, were ineffective in preventing the fall of histidyl-tRNA synthetase specific activity when the preparation was diluted after purification (not shown). In contrast, however, the addition of human hemoglobin at 1.25 mg/ml not only partially preserved the enzyme activity when the enzyme was diluted, but actually stimulated the activity up to two-fold over a wide range of protein concentration (fig.1). The maximum stimulation occurred at a protein concentration of 12.5 μg/ml with a hemoglobin concentration of 1.25 mg/ml.

Hemoglobin over the range of 0–2 mg/ml stimulated the enzyme activity with a maximum of about 6-fold stimulation of very dilute enzyme at 2 mg/ml hemoglobin concentration (fig.2). For demonstration of this optimum stimulatory concentration of hemoglobin, the protein concentration used was 10 μg/ml. Above 2 mg/ml hemoglobin concentration there was a diminution of the stimulatory effect (fig.2). Although purified human globin protein was ineffective, freshly prepared hematin could substitute for hemoglobin,

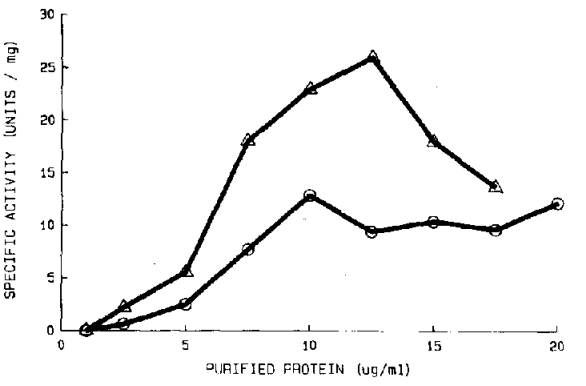

Fig.1. Effect of hemoglobin on enzymatic activity of histidyl-tRNA synthetase. Histidyl-tRNA synthetase activity was assayed in the absence (○) or presence (△) of 1.25 mg/ml hemoglobin.

and furthermore stimulated HeLa histidyl-tRNA synthetase activity even more strikingly (fig.2). Unlike the stimulatory role of hemoglobin, where there was a peak for the stimulation effect, the hematin effect appeared to be an all-or-none phenomenon. Below 0.4 mg/ml it had no effect, but above that concentration there was a plateau of enzyme stimulation. At 0.4 mg/ml the molar concentration of hematin was 6×10^{-4} for 5.5-fold stimulation of the enzyme (fig.2), and the enzyme concentration was approx. 1×10^{-8} M.

The effect of hemoglobin on the stability of the

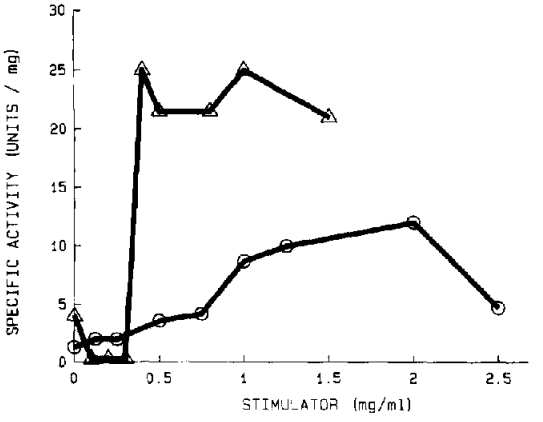

Fig.2. Stimulation of HeLa histidyl-tRNA synthetase using 10 μg/ml protein concentration by hemoglobin or hematin. Histidyl-tRNA synthetase activity was assayed either with the addition of hemoglobin (○) or hematin (△) in the amount indicated.

Table 1

Stabilization of enzyme by hemoglobin stored at −80°C

	Specific activity (units/mg)	
	Day 0	Day 3
Enzyme alone	12.42	0.083
Enzyme with hemoglobin	22.77	9.911

Diluted protein concentration for storage (at −80°C) was 10 μg/ml. Hemoglobin concentration added to the enzyme for stabilization was 1.5 mg/ml prior to freezing

enzyme stored at −80°C was then tested. When the enzyme was stored at 10 μg/ml (protein concentration) immediately after preparation (an elution from the HPLC-DEAE column by 0–300 mM monobasic potassium phosphate gradient), less than 1.0% of the original activity remained after 72 h. If hemoglobin was added immediately before storage, 40% of the original activity was present at 72 h (table 1). Furthermore, when enriched HeLa histidyl-tRNA synthetase was stored at 20 μg/ml for 7 days at −80°C in the absence of hemoglobin, a stimulatory effect of hemoglobin added at the time of assay (at 1.25 mg/ml) could be detected.

4. DISCUSSION

Destruction of proteins can occur in a variety of ways, including direct fragmentation caused by oxygen radicals [8] and peptide bond hydrolysis caused by proteolytic enzymes. Oxygen radicals cannot only cause direct fragmentation, but can alter protein structure so as to increase susceptibility to proteolytic digestion [9]. A major pathway of proteolysis involves the protein ubiquitin and ATP [10–12].

Because ATP was continuously present during purification and assay of the enzyme, and because the enriched enzyme was still accompanied by unidentified proteins, we considered the possibility that a ubiquitin-dependent destruction accounted for the loss of enzymatic activity upon dilution or storage. This possibility was enhanced by the observation that hemoglobin and, particularly, hematin, which may inhibit ubiquitin-related proteolysis [13], protected against loss of enzymatic activity. Ubiquitin (50 μg/ml) added in the presence of 6 mM 5′-ATP, however, failed to inhibit enzymatic activity at earlier steps of purification (unpublished).

Stoichiometric binding of hematin to the enzyme is unlikely because a great molar excess of protector to enzyme is required for the effect. However, previous work has indicated that oxygen radicals may be responsible for initiating protein damage [8,14,15] and hemin can markedly decrease the degradation of oxidatively damaged protein [9]. Other studies have shown that globin and hemoglobin could themselves be fragmented and aggregated by exposure to different oxygen radicals [8]. Together, these observations are in accord with our findings that hematin, and to a lesser extent hemoglobin, but not globin itself can stimulate and stabilize HeLa histidyl-tRNA synthetase. This approach can be applied to stabilization and stimulation studies of other enzymes.

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