Biochimica et Biophysica Acta, 320 (1973) 397–405

BBA 27220

IRON–ATP, A BY-PRODUCT OF ACID EXTRACTION OF WHOLE BLOOD OR RED BLOOD CELLS

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(Received March 19th, 1973)

SUMMARY

Trichloroacetic acid extracts of red cells often produce an iron–ATP* complex after ion exchange chromatography of the extract amounting to about 1/3 of the total ATP. In the present work the presence of 14–50 % of iron–ATP in such extracts from human and Rhesus monkey blood has been shown.

Experiments designed to clarify the possible role and origin of iron–ATP revealed that non-acid treatment of human whole blood or red cells, as in the freeze–thaw process, followed by separation on a Sephadex column did not produce an iron–ATP fraction. In addition, purified hemoglobin and ATP were combined and incubated at pH 7.4. After Sephadex chromatography, there was no evidence of an iron-ATP fraction. However, similar combinations of incubated hemoglobin and ATP treated with trichloroacetic acid and separated by ion exchange chromatography did produce an iron–ATP fraction similar to that obtained from acid-extracted blood.

It appears that iron–ATP in quantities found in acid-extracted blood is the result of iron release from hemoglobin and the subsequent complexing of such iron with available ATP.

INTRODUCTION

For many years an iron adenosine 5′-triphosphate (iron–ATP)* complex has been reported when whole blood or red blood cells were extracted with trichloroacetic acid in studies of the soluble nucleotides and other glycolytic intermediates[1,2]. After ion exchange chromatography, this material, sometimes called "AXP" appeared as a single peak or overlapped the trailing edge of the much larger ATP curve when eluted with a linear gradient of formic–formate buffer[3]. Investigators have stated that 20–50 % of the ATP obtained from such acid extracts of whole blood or red cells is in the form of an iron–ATP complex[4].

* We are using the term, iron–ATP for compounds reported as "AXP" and $(Fe)_x(ATP)_y$.

The iron–ATP was said to be more stable during blood bank storage, its shelf life being at least twice that of ATP[5]. In the postulated complex $(Fe)_x(ATP)_y$ the ratio of x:y has been given varying values, while others have concluded that iron–ATP was a mixture of two or more compounds[6]. However, no physiological role has been demonstrated for iron–ATP, whose appearance has always been associated with acid extraction of whole blood and red cells[5].

If about 1/3 to 1/2 of the red cell ATP was indeed bound to iron, this should command closer study to understand the conditions under which it is formed and its role in red cell metabolism. From such studies, important inferences might be drawn for its role in glycolytic regulation and energy metabolism in the cell. This area is of obvious importance in view of the important relationship between red cell metabolism and oxygen transport[7].

In the course of our investigations on the effect of exercise, hormones, and hypoxic stress on human and monkey red cell metabolism, our attention was drawn to the ATP and iron–ATP fractions after trichloroacetic or perchloric acid extraction of blood. We were puzzled by the varying yields and other anomalous aspects of this complex. We, therefore, designed a series of experiments to study the conditions under which iron–ATP is formed and its possible role in the metabolic pathways of the red cell.

MATERIALS AND METHODS

Adenosine 5′-triphosphate, the crystalline disodium salt, prepared by a non-acid extraction from equine muscle was obtained from Sigma, Sephadex was obtained from Pharmacia. Partially purified and concentrated hemoglobin was prepared as previously described[8]. AG 1X-8 resin, formate form, 200–400 mesh came from Bio-Rad Labs, and was treated to remove material absorbing at 260 nm, with 1 M formic acid prepared in 50 % absolute ethanol and by washing 5–6 times with deionized distilled water. All other chemicals were reagent grade.

Analytical procedures

Hemoglobin and microhematocrits were determined by standard hematologic methods. Enzymatic assay of 2,3-P_2-glycerate and ATP from trichloroacetic acid blood extracts were carried out as previously described[9,10]. Iron was analyzed colorimetrically[11] and by atomic absorption spectroscopy[12] by two independent laboratories according to standard procedures. All adenine-absorbing fractions were determined at pH 2 in a Gilford spectrophotometer at 260 nm. Phosphate was assayed by the Bartlett (a modified Fiske–Subbarow) method[2] with special adaptation to assay large numbers of samples quickly and safely.

Ion exchange chromatography: preparation of the sample by acid extraction techniques

A. Trichloroacetic acid. Freshly drawn heparinized blood (60 ml human or 30 ml monkey) was centrifuged immediately at $10\,000 \times g$ for 10 min, separated and washed once with physiological saline. 20 ml of human or 10 ml of monkey packed red cells from which aliquots had been removed for base line assays (hemoglobin, hematocrit, ATP) were reserved for acid extraction of the nucleotides and other metabolic intermediates.

B. Perchloric acid. 20 ml of human red cells were homogenized in cold 0.6 M $HClO_4$ by the Minakami method as modified in this laboratory[13]. Because of the greater viscosity of red blood cells as compared to whole blood, the acid ratio was increased from 2:1 to 3:1. After removal of excess perchlorate, residual carbonate was flushed out as CO_2 with 1 M HCl. The final extract was diluted to 100 ml with distilled water before applying to the column.

Ion exchange chromatography

The Bartlett procedure[14] for separation of the nucleotides and glycolytic intermediates of an acid extract of red blood cells was used. ATP and iron–ATP are separated from other metabolites by gradient elution with 0–4 M formic–formate (acid–salt, 4:1, v/v, pH 3). In this system the ATP appears after 100 fractions. 10-ml fractions were collected at a drop rate of 1 ml/min and required 35–40 h to separate the desired metabolites.

Samples for iron analysis were collected by pooling aliquots from each fraction representing the desired peak obtained after plotting the adenine absorption at 260 nm. Values were obtained for ATP, iron–ATP, and the sum of the two compounds, and compared with controls.

Sephadex column chromatography: sample preparation by non-acid techniques

For whole blood samples, 10 ml of venous heparinized blood from which the usual aliquots were taken for base line assays were subjected to freeze–thaw lysis 3 times in an acetone–solid CO_2 bath, and centrifuged for 30 min at 3 °C, 45 000 × *g*, and 4 ml of the supernatant were reserved for chromatography.

Packed red cells were obtained as previously described. Samples were diluted 1:1 with the elution buffer, lysed by the freeze–thaw process and clarified by centrifugation as with whole blood. 4 ml of the supernatant were used for Sephadex separation.

Chromatography with Sephadex. The resin, G-25 fine, was equilibrated with the eluent, 0.01 M Tris buffer in 0.145 M saline, pH 7.4, a slurry poured to form a column 75 cm × 1.5 cm and the sample was applied underneath a thin layer of the eluent on top of the resin by means of a bent pipet.

4-ml fractions were collected at a rate of 1 ml/min. When the hemoglobin color was eluted from the column, the nucleotides appeared rapidly in the colorless fractions and these were monitored by reading the absorption values at 260 and 280 nm. These ratios were compared to values obtained from an ATP standard solution. When zero readings appeared, two or more void volumes were passed to assure complete elution; such separations required about 2 h. Iron analyses were made on the total fraction containing adenine-type nucleotides (AMP, ADP, ATP) which were not separated as in ion exchange chromatography.

Experiments combining purified hemoglobin and commercial ATP

A. Non-acid treatment. 1 ml of a 6 μM ATP solution in Tris buffer, 0.01 M in physiological saline and 1 g hemoglobin in 3 ml saline were mixed and aerated by repeated inversion. After 1 h at 0 °C, the mixture was chromatographed on a Sephadex column. Iron analyses were made on the total adenine-absorbing fraction.

B. Acid extraction. 2 ml of an aqueous 6 μM ATP solution and approximately 1 g hemoglobin in 30 ml physiological saline were combined, mixed, aerated, and

incubated for 10 min at 0 °C. The mixture was extracted by the trichloroacetic acid method and separated by ion exchange chromatography as described for blood extracts, and iron analyses determined on the total adenylate fraction. About 15 % of the original ATP was converted to iron–ATP in the short incubation time.

Synthesis of crystalline iron–ATP from iron perchlorate and commercial ATP

Iron–ATP was prepared according to the method of Goucher and Taylor[15]. For purposes of isolating the crystalline iron–ATP, best results were obtained by using concentrated solutions and small volumes. When 60 μmoles each of ATP and iron perchlorate were combined in a final volume of 4 ml of 0.01 M $HClO_4$, pH 2, with rapid stirring, a dense white precipitate formed immediately. After 2 h at 0 °C, the crystalline material was washed by centrifugation 3 times, using small amounts of cold 0.01 M $HClO_4$. The crystals were filtered, dried in vacuum (0.01 mm Hg) and observed under crossed polarizing lenses of the microscope. The long rectangular prisms were anisotropic, and extinguished 4 times in 360 ° rotation of the circular stage. The crystals were used to prepare a solution of known concentration from which iron content was determined, and ATP analyzed enzymatically.

RESULTS

After trichloroacetic acid extraction of packed red cells followed by ion exchange chromatography, human and Rhesus monkey values of iron–ATP as shown by iron analyses, varied from 14–50 % in 14 experiments. Initially, these experiments were done to determine if hypobaric exposure of monkeys (0.5 atm, 48 h) or human exercise (treadmill, 7 mile/h, 0 grade, up to 60 min) affected the level of iron ATP. There were no consistent effects or amount of iron–ATP obtained in the extract after these stresses. The data are pooled in Table I, and confirm earlier work that, in blood extracted with trichloroacetic acid, a significant proportion of ATP is bound to iron. The percentage ATP present as iron–ATP varied unpredictably among the samples, but the sum of ATP *plus* iron–ATP showed consistent agreement with the value for total ATP as found from enzymatic ATP assay (Table I).

TABLE I

ATP AND IRON–ATP FROM HUMAN RED BLOOD CELLS AFTER TRICHLOROACETIC ACID EXTRACTION AND ION EXCHANGE CHROMATOGRAPHY, COMPARED WITH ENZYMATIC ASSAY OF ATP

The results are expressed as μmoles/ml red blood cells. The values represent the means ±S.D.

Subject	*Number of experiments*	*Trichloroacetic acid extraction, ion-exchange fractions*			*Enzymatic assay of ATP*
		ATP	*Iron–ATP*	*ATP+iron–ATP*	
Man	8	0.862 (0.184)	0.363 (0.134)	1.230 (0.091)	1.180 (0.115)
Monkey	6	0.905 (0.089)	0.318 (0.069)	1.223 (0.120)	1.220 (0.196)

Fig. 1 shows a complete ion exchange chromatogram of the Rhesus monkey typical of six experiments and closely resembling that of human subjects. The large

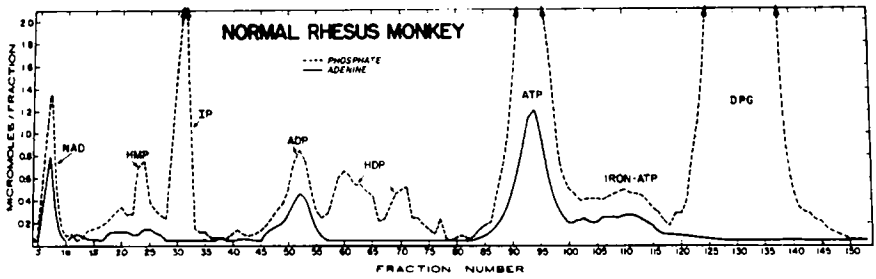

Fig. 1. Typical chromatogram of blood from a normal Rhesus monkey after trichloroacetic acid extraction and separation of the glycolytic intermediates by ion exchange chromatography. The ATP and iron–ATP curves appear between Fractions 85–120 similar to chromatograms obtained with human blood. (Other peaks are the usual red blood cell metabolites[2].)

ATP curve and the small adjacent one for iron–ATP are shown as they appear after gradient elution with 4 M formic–formate buffer, pH 3.

When, however, either whole blood or red blood cells were lysed by the freeze–thaw process, and the proteins separated from the adenine fractions by gel filtration in a Sephadex column, no significant amounts of iron could be detected in the adenine-absorbing fraction. Table II is representative of the difference in the values for iron obtained from non-acid treatment (seven experiments) of the blood sample as compared with a trichloroacetic acid extraction, when a human sample was divided for simultaneous analysis by both methods as well as by enzymatic assay. Here, too, the values for total adenine nucleotides by both methods compare favorable with enzymatic values. Removal of protein from a blood sample is necessary before ion exchange chromatography, so that a simple freeze–thaw hemolysate cannot be applied directly to the ion resin as in gel filtration with Sephadex.

TABLE II

TYPICAL FREEZE–THAW LYSIS OF HUMAN RED BLOOD CELLS AND SEPHADEX COLUMN SEPARATION OF THE HEMOLYSATE COMPARED TO SIMULTANEOUS TRICHLOROACETIC ACID EXTRACTION AND ION EXCHANGE CHROMATOGRAPHY OF AN ALIQUOT OF THE SAME SAMPLE

ATP and iron–ATP values are compared from both methods and with enzymatic ATP analysis. Normal male subject. The results are expressed as μmoles/ml red blood cells.

Method	*AMP*	*ADP*	*ATP*	*Iron–ATP*	*Total adenylate fraction*	*Total iron associated with adenine nucleotides*
Acid extraction, ion exchange	0.03	0.22	1.0	0.3	1.5	0.32*
Enzymatic assay	0.02	0.19	1.2		1.44	
Freeze–thaw, Sephadex separation					1.4	0.09

* Background = ±0.05

Comparative studies in which purified hemoglobin and ATP were combined and incubated yielded no iron-containing fractions after Sephadex chromatography.

(In a typical experiment, expected iron was 4.5 μmoles; found, 0.06 μmoles where ATP was the limiting factor.) However, when such mixtures were subjected to acid extraction and ion exchange separation, an iron–ATP fraction was isolated along with recovered ATP as shown in Fig. 2a. The peaks were remarkably similar to those obtained from ion exchange chromatography of acid-extracted whole blood or red cells (Fig. 2b).

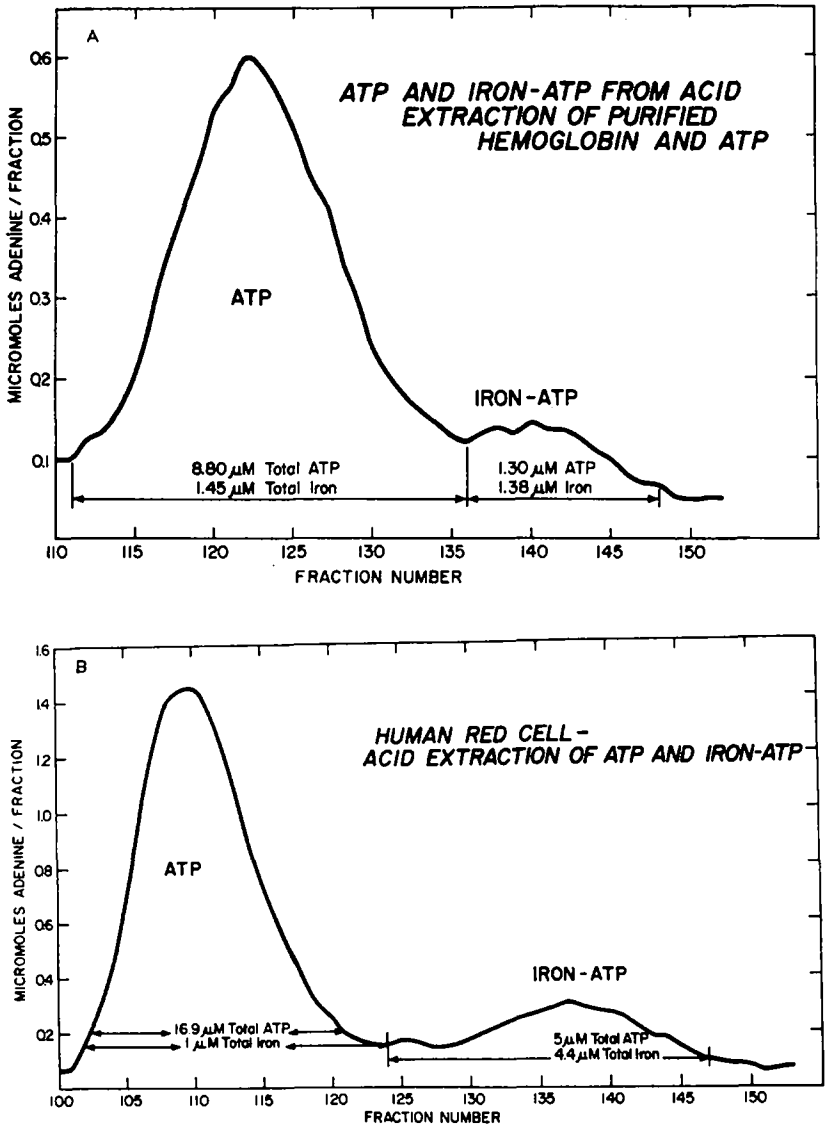

Fig. 2. After ion exchange chromatography of the acid extract, ATP and iron–ATP were found after 110 fractions had been eluted (A) similar to results obtained from acid-extracted blood (B).

$HClO_4$ extraction of red cells resulted in an ion exchange pattern of glycolytic in rmediates similar to that obtained from trichloroacetic acid extraction, but the yi ds of iron–ATP were 1/3 lower than usual. This apparently is due to the loss of iron during the removal of excess $HClO_4$ by means of K_2CO_3 in the presence of triethanolamine buffer. The relatively insoluble $KClO_4$ is centrifuged for removal carrying with it some of the coprecipitated iron salts. In the trichloroacetic acid type of extraction, the excess acid is removed at a low pH by means of ethyl ether in which it is soluble, and iron is not lost.

The role of CO_3^{2-} in removing iron was explored in a series of comparison tests. Trichloroacetic acid extracts of whole blood or red blood cells which gave the usual values of iron (iron–ATP) showed negligible amounts of iron after standing 0.5 h over insoluble $MgCO_3$. Normal iron assay was 0.27 μmoles/ml whole blood; after standing 30 min over $MgCO_3$, 0.09 μmoles/ml whole blood. (This method is used routinely in serum iron analysis to scavenge loosely-bound iron.)

When iron–ATP was synthesized from ATP and iron perchlorate, the ATP was still available to enzymatic analysis in agreement with previous observations[15]. Assay of the iron–ATP with the enzymatic procedure which used yeast hexokinase indicated that the material reacted as did a standard solution of ATP. The data confirm the formula of a 1:1 ratio of iron to ATP in a solution prepared from the purified crystals; calculated for (Fe) (ATP) μmoles/ml: iron, 1.75, adenine, 1.78; found: 1.84, and 1.9.

DISCUSSION

In our laboratory, $HClO_4$ extraction of blood also produced an iron–ATP fraction as well as the spectrum of curves usually obtained with trichloroacetic acid and ion exchange chromatography. However, in a non-acid medium such as in the freeze–thaw process*, the lysate of either whole blood or red cells did not show any iron in the adenylate fraction separated from proteins by Sephadex chromatography. Nor was iron–ATP obtained from combining purified hemoglobin with ATP followed by Sephadex chromatography. But when such a hemoglobin–ATP mixture was treated with trichloroacetic acid as in the usual mammalian blood extraction, iron–ATP was found by ion-exchange chromatography. Fig. 2a shows that the yield and ratio of iron to ATP was similar to that found from acid-extracted human blood (Fig. 2b).

It would seem that iron–ATP is a product of strong acid extraction of whole blood or red cells (a 6.7 % solution of trichloroacetic acid has a pH of about 1). It is probably formed from the freeing of iron from hemoglobin in acid solutions in the presence of ATP. There is considerable evidence for the strong affinity of ATP for complexing with transition elements in various combinations[16,17]. This property is undoubtedly responsible for the formation of iron–ATP in the amounts observed after acid extraction of blood. In the red cells, free iron is reported as 2.5 mg/100 ml and such a small amount would not account for the yields of iron–ATP generally observed.

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* Presumably other types of non-acid lysis such as water, and sonication would show similar results. Other appropriate types of chromatography may also be selected.

The determination we commonly use for ATP is a coupled assay involving hexokinase and glucose-6-phosphate dehydrogenase[18]. The usual substrate for hexokinase is a magnesium–ATP complex and magnesium is included in the assay. The observation that iron–ATP is recoverable here indicates either that magnesium is capable of displacing the iron in the complex or that iron–ATP itself may serve as a substrate. Most assays for ATP in blood begin with an acid extraction to remove proteins, in which process iron–ATP has inevitably been formed.

Some investigators have reported that the iron–ATP fraction they obtained from acid-extracted blood after ion exchange chromatography was a mixture of compounds due to various iron or phosphate ratios[6]. Further insight into the reasons for the large number of formulas attributed to iron–ATP, $(Fe)_x(ATP)_y$ comes from the laboratory synthesis of the compound with the 1:1 ratio (prepared from iron perchlorate and ATP). This material precipitates at pH 2 and it is highly unlikely that these precise conditions occur in the red blood cell. It has been observed that upon standing and at higher pH, iron–ATP 1:1 forms an equilibrium mixture with a series of compounds of varying iron ratios which are far more soluble.

Since it is acid extraction which frees hemoglobin iron, and in the presence of ATP causes the formation of iron–ATP found by ion exchange chromatography, there is probably no biological significance to be attached to the levels of the compound revealed by acid extraction techniques.

ACKNOWLEDGEMENTS

This work was supported in part by Grant No. 450 from the Michigan Memorial Phoenix Project and the Research and Development Command of the Army and Navy, under Contract DADA17-69-C-9103. We thank Ms Lucia Brewer for valuable technical assistance.

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