MOBILIZATION OF IRON FROM RETICULOCYTES

Identification of pyridoxal isonicotinoyl hydrazone as a new iron chelating agent

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1. Introduction

Incubation of immature erythroid cells with inhibitors of heme synthesis, e.g., INH, and transferrin-bound ^{59}Fe results in iron accumulation in various non-heme compartments including mitochondria [1–3]. Recently we isolated ^{59}Fe-labeled mitochondria from reticulocytes preincubated with INH and ^{59}Fe-transferrin. Reticulocyte lysate was able to mobilize iron from the mitochondria and this iron release was significantly stimulated by pyridoxal-5-phosphate [4].

In experiments presented here we observed that the addition of pyridoxal-5-phosphate or pyridoxal to ^{59}Fe-loaded reticulocytes incubated with INH to block ^{59}Fe utilization for heme synthesis caused the release of a considerable amount of radioiron from the cells. We demonstrated that this mobilization was due to the presence of a hydrazone formed from INH and pyridoxal or pyridoxal-5-phosphate. Moreover, pyridoxal isonicotinoyl hydrazone was shown to form a complex (chelate) with iron in the solution.

2. Materials and methods

2.1. *Chemicals*

INH was obtained from Spofa, Prague, pyridoxal hydrochloride and pyridoxal-5-phosphate from Merck, Darmstadt. Desferrioxamine was kindly provided by Ciba-Geigy, Basel. ^{59}Fe-citrate (10 mCi/mg Fe) was purchased from the Zentralinstitut für Kernforschung, Dresden. Incubation medium [5], which is similar to M 199, was obtained from the Institute of Sera and Vaccines, Prague.

2.2. *Preparation of pyridoxal isonicotinoyl hydrazone (PIH) and PIH–iron complex*

Pyridoxal isonicotinoyl hydrazone was prepared in good yield by mixing together in equimolar quantities nearly saturated solutions of INH and pyridoxal, both in 0.1 M sodium acetate buffer (pH 4.5) and heating the mixture for 2–3 min at 100°C. The relatively insoluble condensation product was filtered from the cooled solution, washed with water and dried. The hydrazone is well soluble in dilute mineral acids and when adjusted to pH 7.4 it does not precipitate at concentrations of approx. 5 mM or less.

A complex of pyridoxal isonicotinoyl hydrazone with iron was obtained by mixing together freshly prepared solutions of PIH (5 mM, in 50 mM Tris, pH 7.4) with 3 mM ferric citrate (1 mol Fe^{3+} : 10 mol citrate, pH 7.4). The dark red-brown precipitate, which was formed immediately after mixing both solutions, was filtered, thoroughly washed with water and dried. Before addition to the incubation mixture, PIH–Fe was mixed with a buffered salt solution (see below), dissolved by adding a small volume of 0.15 M HCl and adjusted to pH 7.4 with NaOH.

Abbreviations: INH, isonicotinic acid hydrazide; PIH, pyridoxal isonicotinoyl hydrazone; Tris, Tris–(hydroxymethyl)aminomethane

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2.3. *Reticulocyte preparation and incubation*

Reticulocytes were obtained from phenylhydrazine-treated rabbits [6] and washed prior to use as in [1].

^{59}Fe-labeled rabbit plasma was prepared as in [2].

Basic experimental design was similar to that in [1] with certain modifications. Reticulocytes were pre-incubated with INH (10 mM) and ^{59}Fe-labeled rabbit plasma for 60 min and thoroughly washed using the procedure in [1]. After this treatment radioiron accumulates in mitochondria (40–50% total cellular radioiron), in a low molecular weight fraction (25–30%) and ferritin (10–15%) and only the remaining 15–10% total cellular ^{59}Fe is found in hemoglobin [2]. In the second phase of the experiment, ^{59}Fe-labeled reticulocytes (approx. 20% suspension) were reincubated in a buffered salt solution [7] in a humidified atmosphere at 37°C in plastic tubes. The final volume of the incubation mixture was 1 ml. The volume of incubated reticulocytes was calculated from the packed cell volume determined by the microhematocrit method. At indicated time intervals the tubes were transferred to an ice-bath and the cells and supernatant solution of each sample were separated by centrifugation at 1500 × *g* for 10 min at 4°C. Cells were washed twice with cold saline. The radioactivity was counted [1] in both reticulocytes and in an aliquot of the supernatant and the percentage of iron mobilization from reticulocytes was calculated.

The degree of hemolysis during reincubation was monitored by measuring A_{545} of an aliquot of the supernatant to which KCN was added [8]. Those experiments in which cyanmethemoglobin was detected in the supernatant were not taken into consideration.

2.4. *Analytical methods*

Specific activity of heme and the % total cellular radioiron in heme were determined as in [1]. Spectra were recorded using a Unicam SP-1 700 spectrophotometer.

3. Results and discussion

During 120 min reincubation of ^{59}Fe-labeled reticulocytes in Hanks' solution, a maximum of 1.5% radioiron was released from the cells (table 1). Since

Table 1

Effect of pyridoxal-5-phosphate or pyridoxal on iron release from radioiron-loaded reticulocytes reincubated without or with INH (10 mM)

Exp.	Additions	% Iron release from ^{59}Fe-reticulocytes				% ^{59}Fe utilized for heme[a] synthesis during 120 min reincubation
		Incubation time (min)				
		15	30	60	120	
1	Nil (control)	1.2	1.2	1.4	1.5	77.2
	INH	0.9	1.3	1.3	1.3	30.0
	Pyridoxal-5-phosphate (0.1 mM)	1.0	1.7	1.8	2.6	79.1
	Pyridoxal-5-phosphate (1.0 mM)	1.7	1.8	2.4	3.8	83.6
	INH + pyridoxal-5-phosphate (0.1 mM)	1.3	1.6	2.7	5.2	35.6
	INH + pyridoxal-5-phosphate (1.0 mM)	3.5	5.8	9.2	20.0	42.5
2	INH	1.2	1.1	1.1	1.1	28.1
	Pyridoxal (0.1 mM)	2.2	2.8	2.9	3.0	73.1
	Pyridoxal (1.0 mM)	3.6	4.3	8.0	8.6	73.4
	INH + pyridoxal (0.1 mM)	9.3	17.1	24.9	30.7	24.1
	INH + pyridoxal (1.0 mM)	27.2	50.0	72.4	77.7	15.1

[a] After 120 min reincubation of reticulocytes, [^{59}Fe]heme radioactivity was determined [1] and expressed as a % ^{59}Fe-radioactivity found in reticulocytes (=100%) prior to the second incubation

The results are the means of duplicate samples; the differences between duplicates did not exceed 5%. In both experiments after 60 min preincubation with INH and ^{59}Fe-labeled plasma, heme radioactivity represented 15% of total cellular ^{59}Fe

the presence of radioiron in the supernatant is not due to cell lysis (see section 2), it seems probable that radioiron here represents recycling of iron attached to transferrin molecules which have been released from reticulocytes after donating none or only one of their iron atoms [9].

INH, although it had been shown to form stable complexes with metals [10], did not enhance iron release from ^{59}Fe-labeled reticulocytes. Modest, though significant, stimulation of iron mobilization from reticulocytes was observed after addition of pyridoxal-5-phosphate or pyridoxal. INH presence during reincubation considerably enhanced the effect of both compounds (table 1). Similar results were obtained in 5 additional experiments. The more profound effect of pyridoxal on iron mobilization from reticulocytes may be due either to an easier permeability of red-cell membrane for pyridoxal than for pyridoxal-5-phosphate [11] and/or to the fact that pyridoxal-5-phosphate [12], but not pyridoxal, partially restores the rate of heme synthesis reduced by INH (table 1).

One possible explanation for these observations is that INH blocks the utilization of intracellular non-heme iron for heme synthesis and thus renders intracellular ^{59}Fe available for chelation either by each pyridoxal compound alone [13] or by a Schiff base formed from pyridoxal compounds and intracellular amino acids [14,15]. However, when INH was replaced by other heme synthesis inhibitors, such as 5 mM penicillamine [12] or 20 mM levulinic acid [16], the amount of iron mobilized from reticulocytes decreased to the level observed with pyridoxal alone (not shown). These results suggest that a new compound, formed from pyridoxal (or pyridoxal-5-phosphate) and INH, is responsible for a high degree of iron mobilization from the cells.

Formation of isonicotinoyl hydrazones of pyridoxal [17] and pyridoxal-5-phosphate [18] is known and is confirmed here by absorption spectral analysis (fig.1). At pH 7.4, pyridoxal has 2 maxima at A_{252} and A_{316} and INH 1 peak at A_{265}. Mixing of both solutions is accompanied by a spectral change due to the formation of the hydrazone which has a new A_{375} peak. When ferric citrate is added to pyridoxal isonicotinoyl hydrazone solution, a red-brown colour arises and an iron complex formation is indicated by a new A_{465} peak (fig.1) or A_{475} (fig.2), respectively. Figure 3 shows that at most, 1 mol Fe^{3+} can be bound by 2 mol pyridoxal isonicotinoyl hydrazone.

Table 2 provides direct evidence that pyridoxal isonicotinoyl hydrazone mobilizes ^{59}Fe from reticulo-

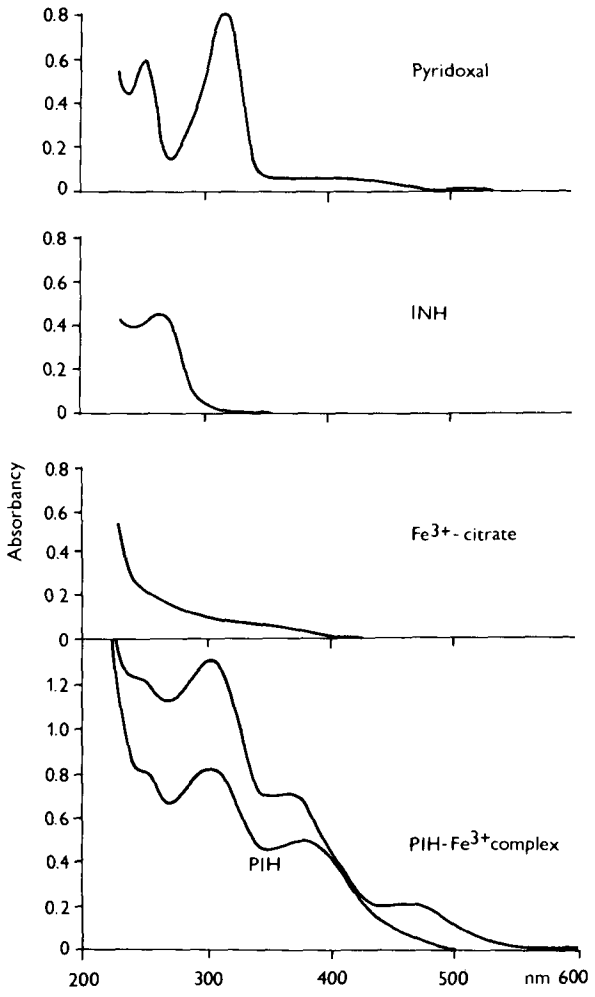

Fig.1. Absorption spectra of pyridoxal isonicotinoyl hydrazone (PIH), PIH–Fe^{3+} complex and individual reagents used for their preparation. PIH–Fe^{3+} complex contained 0.1 mM PIH and 0.033 mM Fe^{3+} in 50 mM Tris–HCl buffer and 10 mM citrate-Na_3 (pH 7.4). PIH was 0.1 mM, pyridoxal 0.1 mM and INH 0.1 mM (all in 50 mM Tris–HCl buffer (pH 7.4)), and Fe^{3+} 0.05 mM. The Fe^{3+} solution was prepared using $FeCl_3 \cdot 6\ H_2O$ in which the amount of iron was determined and this solution was adjusted to pH 7.40 in the presence of 10 mM citrate-Na_3 and 50 mM Tris–HCl buffer (pH 7.40). Spectra were recorded against distilled water as blank.

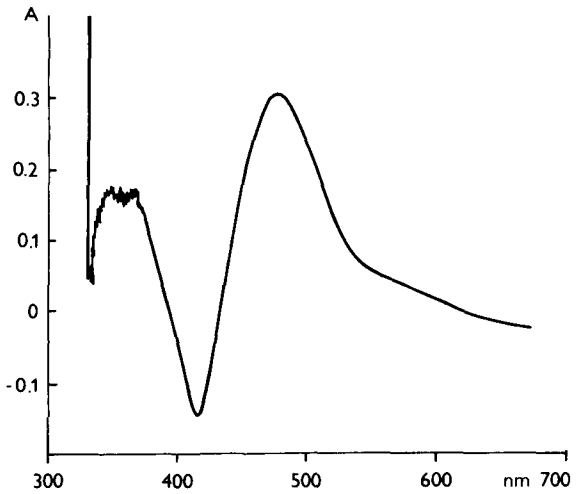

Fig.2. Difference spectrum between PIH–Fe^{3+} complex and pyridoxal isonicotinoyl hydrazone (PIH) and Fe^{3+} solutions. PIH–Fe^{3+} complex contained 0.5 mM PIH and 0.083 mM Fe^{3+} in 50 mM Tris–HCl buffer, 10 mM citrate-Na_3 (pH 7.40). Spectrum was recorded in a 1 cm cuvette against 0.5 mM PIH and 0.083 mM Fe^{3+} in the same buffer–citrate solution, each in a 1 cm cuvette. The solution of Fe^{3+} was prepared as in fig.1.

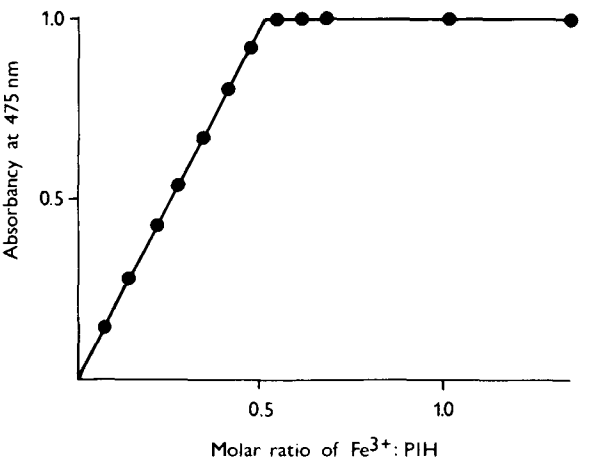

Fig.3. Formation of PIH–Fe^{3+} complex followed by increase A_{475}. The reaction mixture contained 0.5 mM PIH, 50 mM Tris–HCl buffer, 10 mM citrate-Na_3 (pH 7.40) and increasing amounts of Fe^{3+} (0.033–0.666 mM). A_{475} of samples were measured in a 1 cm cuvette after 4 h against 0.5 mM PIH as blank.

Table 2
Effect of desferrioxamine, pyridoxal isonicotinoyl hydrazone (PIH) and PIH–Fe^{3+} complex on iron release from radioiron-loaded reticulocytes

Additions	Conc. (mM)	Iron release from reticulocytes (%)	% ^{59}Fe utilized for heme[a] synthesis during reincubation
Nil (control)	–	1.4 ± 0.0	60.0 ± 0.1
Desferrioxamine	5	3.5 ± 0.3	39.0 ± 0.1
PIH	0.02	7.6 ± 0.5	33.3 ± 2.8
PIH–Fe^{3+}	0.02	2.3 ± 0.3	28.7 ± 3.8
PIH	0.05	24.6 ± 1.1	34.0 ± 1.5
PIH–Fe^{3+}	0.05	2.7 ± 0.4	36.9 ± 1.0
PIH	0.1	38.6 ± 0.5	29.3 ± 1.2
PIH–Fe^{3+}	0.1	4.1 ± 0.0	33.8 ± 1.2
PIH	1	66.2 ± 0.2	12.6 ± 0.2
PIH–Fe^{3+}	1	6.7 ± 0.4	32.8 ± 0.6

[a] This value was obtained as in table 1 but after 60 min reincubation

The results are means ± SEM of 4 samples. After 60 min preincubation with INH and ^{59}Fe-labeled plasma, heme radioactivity represented 12.5 ± 1.2 of total cellular ^{59}Fe. Radioiron release into the media and ^{59}Fe utilization for heme synthesis were measured after 60 min reincubation of ^{59}Fe-labeled reticulocytes

cytes with particular efficiency; a definite effect is demonstrable already at 20 μM hydrazone. It should be pointed out that 20 μM pyridoxal isonicotinoyl hydrazone mobilizes twice as much iron as 5 mM desferrioxamine. The effects of other iron chelating agents (e.g., nitrilotriacetic acid, 2,2'-dipyridyl, 2,3-dihydroxybenzoic acid, rhodotorulic acid) are quantitatively comparable to that of desferrioxamine. Iron pre-bound to pyridoxal isonicotinoyl hydrazone considerably decreases the mobilization of ^{59}Fe from the cells (table 2). Slight iron release observed in the presence of PIH–Fe^{3+} complex may be due to an exchange of radioiron with unlabeled iron inside the reticulocytes.

Reticulocytes experimentally loaded with non-heme radioiron appear to be a suitable assay system for testing the biological effectiveness of different iron chelating agents.

Our preliminary results indicate that pyridoxal isonicotinoyl hydrazone mobilizes iron mainly from mitochondria of reticulocytes incubated in vitro. Moreover, this agent is an effective in vivo iron chelator able to take up iron from the hepatocyte and excrete it into bile (P. P., E. Nečas and M. Cikrt, to be published). Therefore, pyridoxal isonicotinoyl hydrazone as a biologically effective iron chelating agent seems to require further investigation.

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