5.08J Biological Chemistry II (2016)
ANS Problem Set 9 on iron metabolism

1. Recently studies with mice were carried out to evaluate the effects of a low iron diet with a focus on the duodenal cells. Many of the players important in iron homeostasis were examined using western blot analysis. In one experiment shown in Figure 1A, expression levels of proteins from intestinal mucosa cells (enterocytes) obtained from mice fed a low (L) and high (H) iron diet were compared. In the same experiment, Figure 1B, the levels of the mRNA (Northern blot analysis) were examined. In addition, a similar, but not as extensive, set of experiments was carried out (**Figure 2**) to examine the behavior of Fpn1 during erythroid differentiation (red blood cell differentiation) and whether it is similar to the observations with duodenal enterocytes. Note: Most of our body's iron content is incorporated into hemoglobin during the differentiation of erythroid precursors into mature red blood cells.

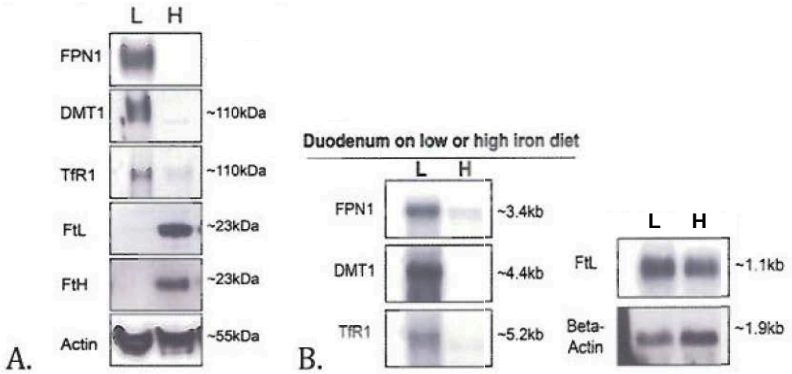

Figure 1A and 1B. **Expression of proteins (A) and mRNA (B)** associated with iron homeostasis in the duodenum (enterocytes, cells involved in iron uptake from the diet) of mice maintained on a low iron diet (L) or a high iron diet (H). **In panel A**, western blots reveal the levels of FPN1 (ferroportin), DMT1 (divalent metal ion transporter), TFR1 (transferrin receptor 1) as well as the levels of the two subunits of Ferritin (FtL and FtH). The actin serves as a loading control. **In panel B,** the mRNA expression levels of mice on a low or a high diet are shown using Northern blot analysis. The data on the far right is part of panel B. The actin serves as the control. The size of the mRNA in kb is shown to the right of each rectangle.

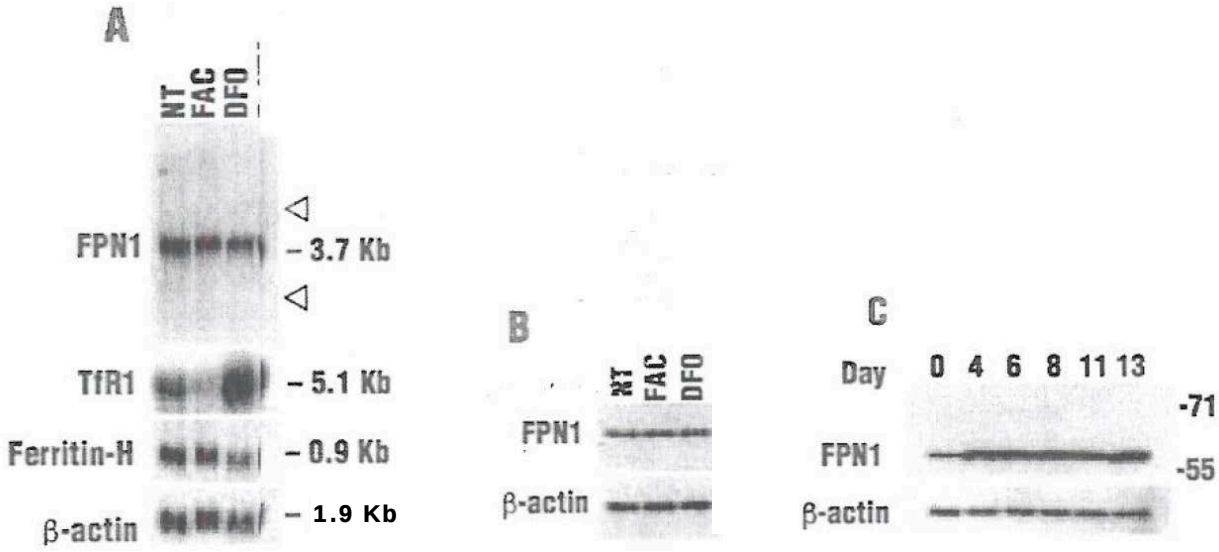

Figure 2. A. Northern blot analysis (monitoring mRNA) of total RNA samples from erythroid cells untreated (NT), and treated with ferric ammonium citrate (FAC) and desferrioxamine (DFO, an iron chelator). B. Western blot where FPN1 is 62 Kda and actin is the loading control. C. Western blot of Fpn1 monitored during the differentiation of the erythroid cells over a period of 13 days.

Questions:

1. Based on the Lectures in Module 6 and the assigned reading, draw the structures of the mRNA of DMT1, TfR1, and one of the ferritin subunits labeling the 5' and 3' ends of their mRNA and clearly indicating the location of the IRE.

2. Rationalize the results shown in Figure 1A and 1B for enterocyte cells when experiencing a diet of low iron or high iron.

Low iron: TfR and DMT1 have 3'-IREs that bind apo-IRP and stablize the mRNA so that protein can be made. Both TfR and DMT1 are involved in iron uptake. For ferritin subunits the IRE is at the 5' end and apo-IRP binding inhibits translation. You still have the mRNA. This result with ferritin (light and heavy chains) is what is expected as at low iron concentrations, the cells do not want to store iron in ferritin.

High iron: apo-IRP1 dissociates from the IRE and can be loaded with 4Fe4S cluster. In this state it can no longer bind to the 5' or 3' IREs. TfR and Dmt1 mRNA can thus become degraded or translated to a lesser extent. This is the result one wants, since iron is plentiful. For ferritin, one would like to store iron if it is in excess. The mRNA for L-ferritin is present at both H and L iron, although only the protein is present at high iron. This mode of reaction involves translation inhibition so this makes sense.

Finally, again remember that regulation is multifactoral with other factors affecting expression levels of proteins in different tissues.

You were not asked about Fpn in this question. However, Fpn is an **iron exporter** and is involved in iron uptake to the plasma from the diet from enterocytes and re-circulation of iron from heme in dead RBCs from macrophages. From the published analysis in these systems Fpn has a 5'-IRE and translation would be prevented at L iron. This is not what is observed with respect to mRNA and protein, in fact the behavior is very similar to Dmt1 and TfR. Thus this data is a reminder that iron homeostasis depends on a coordinated regulation of the expression of molecules involved in import and export. (see figure 2). Recall that a peptide hormone (hepcidin) is a key regulator of iron homeostasis and its major target is Fpn1.

3. The results of similar experiments to those shown in Figure 1 are shown for differentiating erythroids experiencing a diet containing ferric ammonium citrate (FAC) or desferrioxamine (DFO) are shown in Figures 2A-C. Describe and rationalize these results.

The focus is on Fpn. In Fig 2 at H and L iron, the mRNA is apparent in differentiating erythroids. The control in Fig 2 for comparison is the TfR that behaves the same way in this system as in enterocytes. Thus some regulatory mechanism is distinct between the two systems. If you look at the data carefully, you see that the mRNA

for Fpn1 in enterocytes is 3.4 kb, while that in differentiating erythroids is 3.7 kb. This might provide an explanation for the different expression levels of protein and mRNA.

Note: again the regulation is complex and remember hepcidin hormone plays a key regulatory role in Fpn1 levels.

4. Rationalize the differences between the results reported in Figure 1 and in Figure 2 given the physiological function the cell types.
The mRNAs in different tissues might have distinct structures. This could result from different protein binding or altered Kds for IRP binding in the presence of additional proteins or alternative splicing in the two tissues. The latter could remove the IRE so that the mRNA and protein is not affected by IRPs.

2. As you learned in Recitation 10, methods to measure binding constants require some ingenuity and are dependent on the problem at hand. In this problem, a K_d for the interaction between IRBP1 and one specific IRE was determined using a gel shift assay. The IRE is a small piece of RNA made synthetically that has [^{32}P] incorporated at its 5'-end enzymatically. The interaction between the protein and this piece of RNA can be monitored by a gel shift assay (electrophoretic mobility shift assay, EMSA). In this type of assay the RNA-IRP1 complex runs slower on the gel than the RNA alone.

In this assay, a constant amount of 5'- [^{32}P]-IRE oligonucleotide is incubated with varying amounts of IRP1 **in a 100 µL** volume and the products are run on an agarose the gel. For the purposes of this problem, assume the IRP has a molecular weight of 10,000 Da. Once the gel is complete, a phosphorimage is made using the technology described in the recitation on radioactivity. To quantify the observed bands, they are each excised from the gel and analyzed by scintillation counting to determine the amount of radioactivity. A schematic drawing of the phosphorimage is shown in Figure 3 and the counts per min (cpm) found in each gel slice (minus the cpm in a gel slice of the same size with no radiolabled material present, that is the background) are tabullated in Table 1. The specific activity of the labeled RNA is not known exactly, but is in the range of 300 to 3000 Ci/mmol. Recall that 1 mCi = 2.2 x 10^6 decays/min).

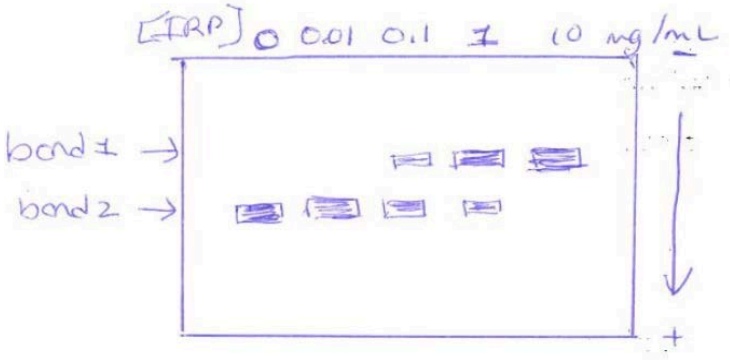

Figure 3. The phosphorimage data (detection of ^{32}P) of the agarose gel. The amount of the IRP is shown at the top of the gel in mg/mL.

Table1: Summary of radioactivity (cpm) associated with band 1 and band 2 from Figure 3

[IRP]	band 1	band 2
none added	-----	9916
10 mg/mL	456	9226
100 mg/mL	3341	6690
1 mg/mL	8167	1703
10 mg/mL	10033	220

Note: band 2 is the IRBP-RNA complex and band 1 is the RNA.

Questions:

1. What type of radiation is released from the ^{32}P nucleus and how does the energy from this species compare with that released from a ^{14}C or ^{3}H-labeled species?

^{32}P as ^{3}H and ^{14}C are all β emitters. The energy of the β particles is shown below, taken from your recitation 8 handout. Thus more precautions need to be taken with ^{32}P than with the more commonly used β emitters due to its higher energy.

Radioisotope	**Emission**	**Half-Life**	**Energy of β (KeV)**
Hydrogen-3 (Tritium)	β	12.3 years	18.6
Carbon-14	β	5730 years	156
Phosphorus-32	β	14.3 days	1710
Sulfur-35	β	87.6 days	167

2. What is the difference between counts per min (cpm) and disintegrations per min (dpm)?

A quantitative measure for radioactivity in a sample is essential. The standard unit of radioactive decay is the curie (Ci), defined as any radioactive substance in which the decay rate is = 3.7×10^{10} disintegrations per second (dps) or 2.2×10^{12} disintegrations per minute (dpm). Because counting efficiency (detection efficiency) is less than 100%, the observed radioactivity is designated as counts per min (cpm). Example: If 1 mCi (2.2×10^{9} dpm) of [^{14}C] acetate is counted (detected) with 50% efficiency, then one observes 1.1×10^{9} cpm. The ^{3}H isotope has the lowest energy and the efficiency of detection often is associated with large quenching effects that is dependent on other materials present during the analysis. Thus the efficiency needs to be carefully assessed with quenching controls.

3. In the experiment described above, calculate the K_d for the interaction between IRP1 and the IRE. To solve this problem you need to think about the amount of $[IRP1]_{total}$, $[IRP1]_{free}$ and $[IRP1 \bullet [IRE]_{bound}$. Look at the notes from recitation 10. [Hint: If you are

having trouble, think about using the information to determine the fraction bound and the additional information you are given.

You are given the concentration of IRP and that it is varied and that the concentration of [^{32}P]-IRE is fixed. In the phosphorimager data, band 2 is the [^{32}P]-IRE and band 1 is the [^{32}P]-IRE•IRP.

You can also calculate the concentration of IRP given the number of µg/mL (see gel) and the molecular weight of 10,000 da. Thus $[IRP]_T$ is 1 µM for 10 µg; 10 µM for 100 µg; 100 µM for 1 mg; and 1.0 mM for 10 mg.

$$[IRP]_T = [IRP]_B + [IRP]_F \quad \text{where } [IRP]_B \text{ is IRP•IRE}$$

As discussed in recitation you need to think about the amount bound and free and what you can actually measure. $[IRE]_T = [IRE]_B + [IRE]_F$

For $[IRE]_T$, for example, you can carry out a crude calculation because you are given that the specific acitivity (SA) of this material ranges between 300 and 3000 Ci/mmol. From Table1 you have 10,000 cpm. Thus let us assume that the SA is 1000 Ci/mmol, an intermediate number between 300 and 3000 Ci/mmol. Given that there are 2×10^{12} cpm/Ci and that you have only 10^4 cpm, you can calculate that you have 5×10^{-15} moles and you know it is in 100 µL. Thus you have 50 pM of IRE. Thus the [IRP] given above, is >>>> [IRE].

You know that $[IRP]_T = [IRP]_B + [IRP]_F$ However, given the above calculation you can now assume that $[IRP]_T$ approx = to $[IRP]_F$

Go to the next two pages (hand written) because of the eqns.

$$K_d = \frac{[IRE]_F [IRP]_F}{[IRP \cdot IRE]}$$

using the above assumptions

$$K_d = \frac{([IRE]_T - [IRE \cdot IRP]) \cdot ([IRP]_T)}{[IRP \cdot IRE]} \quad 1$$

you need to reorganize this eqn(1) into a form that can be analyzed with the available data, From the gel data in Table 1 you can measure the fraction of IRE bound

∴ Reorganize

$$K_d [IRP \cdot IRE] = [IRE]_T [IRP]_T - [IRP]_T \cdot [IRE \cdot IRP]$$

$$\therefore \quad K_d [IRP \cdot IRE] + [IRP]_T \cdot [IRE \cdot IRP] = [IRE]_T [IRP]_T$$

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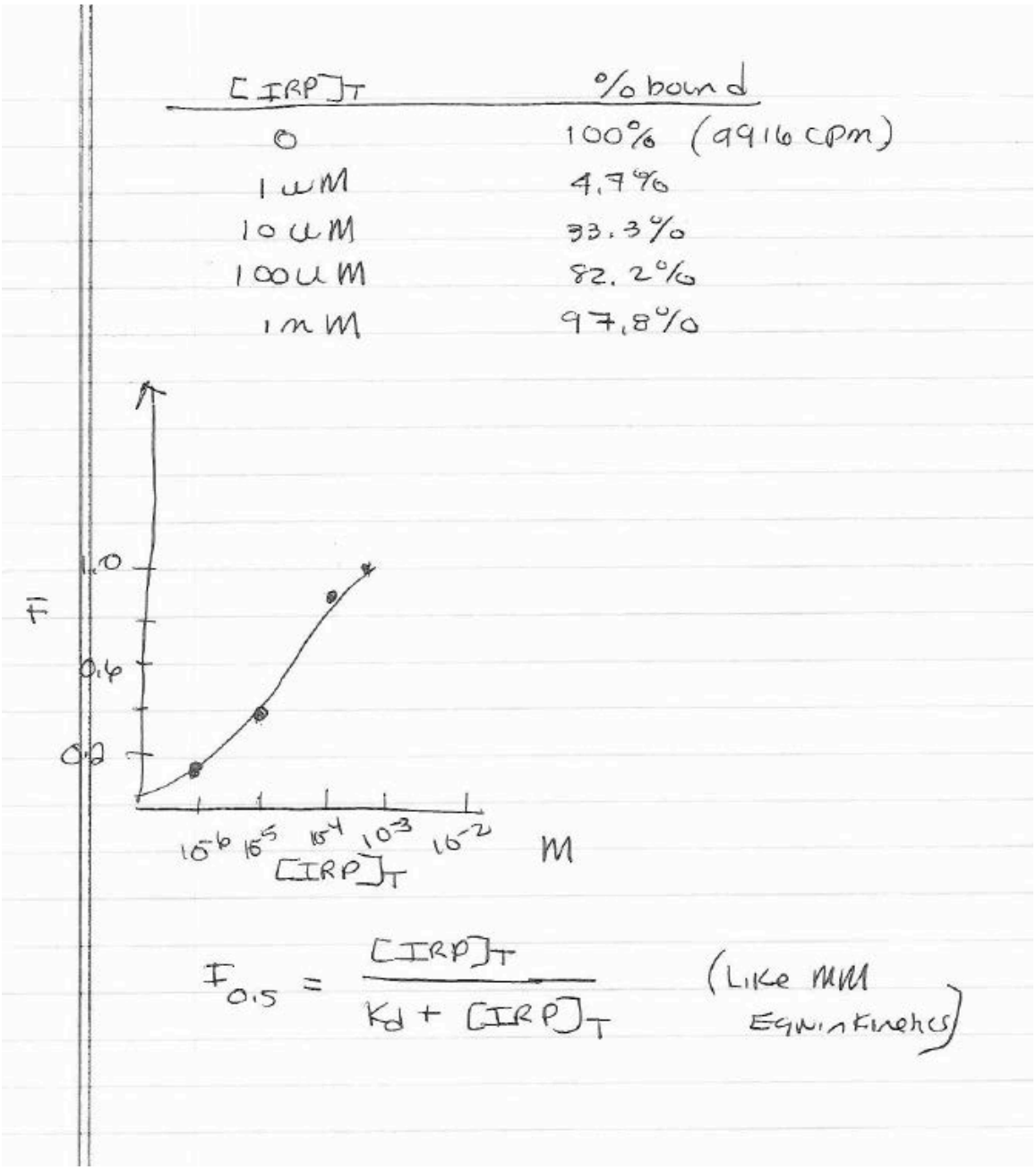
fraction bound is $\frac{[IRE \cdot IRP]}{[IRE]_T} = \frac{[IRP]_T}{K_d + [IRP]_T}$

From the data given



4. What control experiments are required to give you more confidence in your analysis? From the data in the plot it is apparent that you do not have enough data points to provide you with a Kd. Thus you carry out additional experiments with points spanning with equal spacing 0.2 to 5 times the F(0.5) in the above graph.

3. IsdB is critical for the use of hemoglobin (Hb) as an iron source by *S. aureus*, both in vitro (*S. aureus* growth in culture) and during infections in humans. IsdB is the most highly upregulated member of the *isd* gene cluster under all tested iron restricted growth conditions. Its proposed role is to extract the heme from Hb at the cell surface for transfer to IsdA and IsdC. Experiments have recently been carried out to understand which domains of IsdB are necessary and or sufficient for Hb binding and heme uptake

from Hb. To address these problems a variety of constructs of IsdB were generated and are shown in Figure 4.

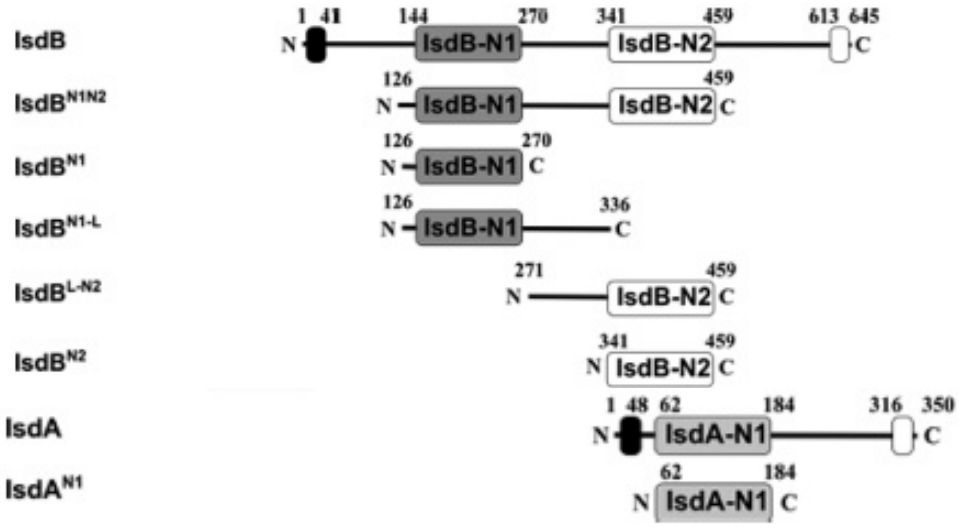


Figure 4 mislabeled in ps as 3. Sequence features of full length IsdB and the recombinant constructs containing the N1, N2 or N1 and N2 NEAT domains that were studied in the experiments described in Figures 5 through 7.

To examine the interaction between Hb (oxyHb and metHb (Fe^{3+}) and IsdB, a $(His)_6$-Hb was constructed and used and the results are shown in Figure 5 not 4.

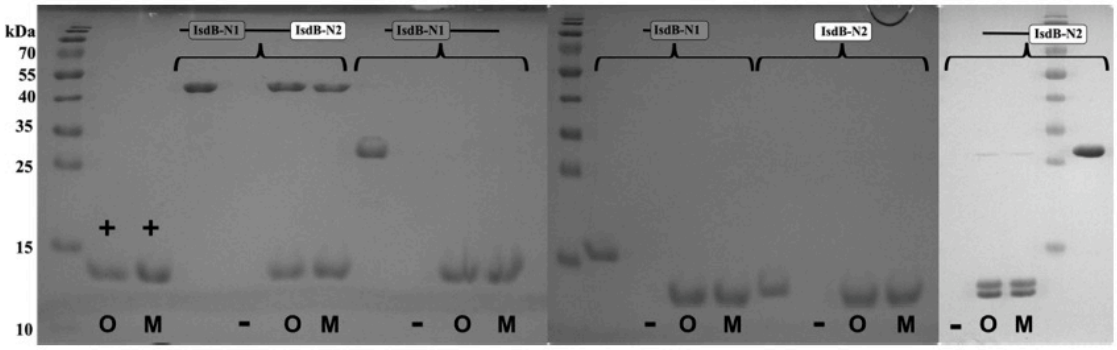


Figure 5. Hb pull-down assay of IsdB constructs. OxyHb (O) and metHb (M) at 20 μM was immobilized on nickel beads, followed by incubation with 20 μM of the distinct IsbB constructs (labels at the top). The bound protein was eluted with 500 mM imdidazole. The positive control (+) shows Hb binding and beads in the absence of IsdB. The negative control (-) shows IsdB in the absence of $(His)_6$-Hb. To the left of each negative control lane is a lane containing one microgram of the indicated IsdB construct.

In a third experiment the authors used visible spectroscopy to determine if heme could be removed from met-Hb (Figure 6).

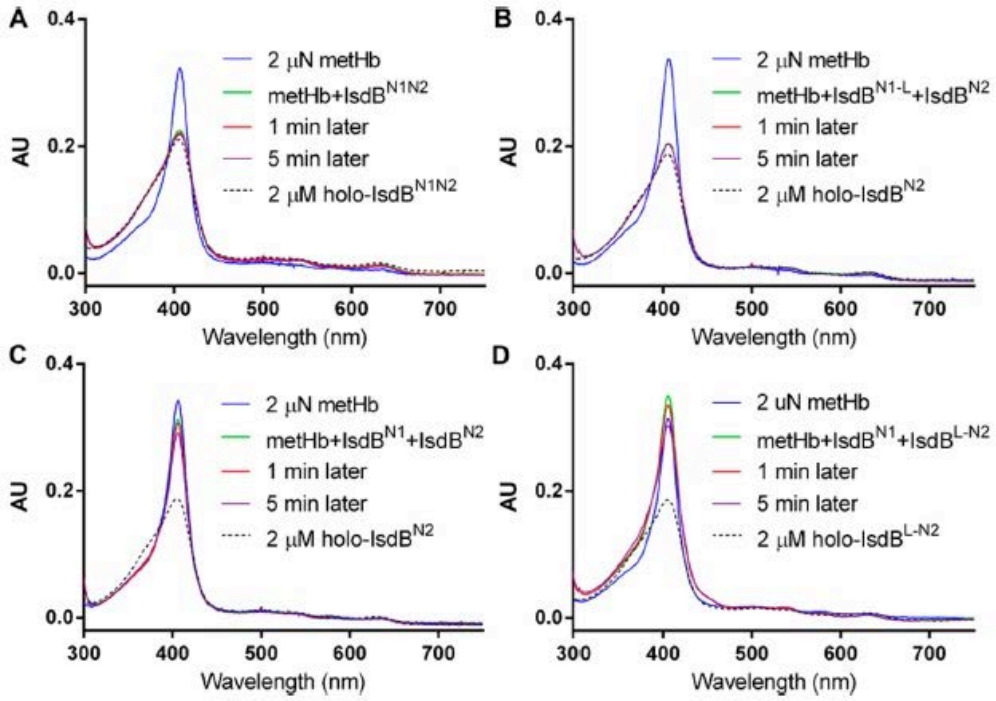


Figure 6. Vis absorption spectra of 2 μM met-Hb mixed with 20 μM of the different IsdB constructs shown in Figure 4. A decrease in the magnitude of the Soret band (400 nm) is associated with heme transfer.

In a final experiment (Figure 7), the authors examined the kinetics of the transfer of heme from metHb to IsdA^{N1} by IsdB^{N1N2}.

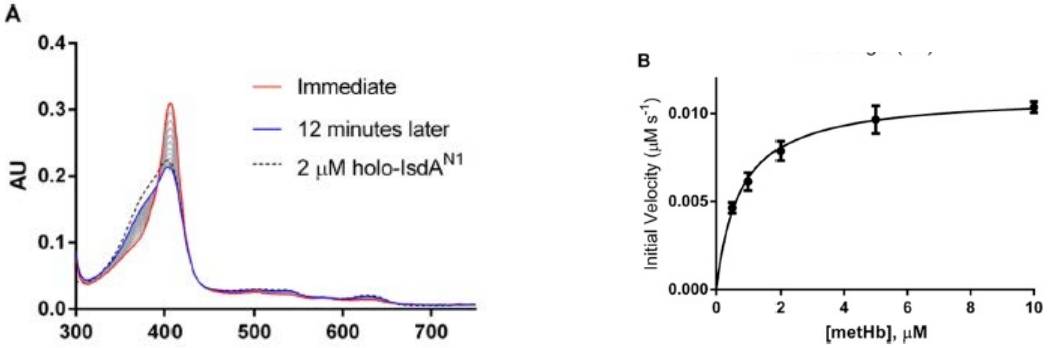


Figure 7. A. Spectral changes observed on incubation of 2 μM met Hb, 50 μM IsdA and 50 nM IsdB. Data for heme reconstituted IsdA^{N1}, diluted to 2 μM B by heme concentration - - - are shown for comparison. B. The experiments in A were repeated except with different concentrations of metHb (1 to 10 μM) and the kinetics were carried out as in A. The results of initial rates for each concentration of metHb were monitored.

Questions: Taken from *Biochemistry* 53, 2286-94 (2014)

1. What does the data in Figure 5 tell you about the form of IsdB required in the extraction/transfer process from Hb? See Figure 4 for construct names.

The data in Figure 5 tells us that the full length ($IsdB^{N1N2}$) construct is needed for the heme extraction activity of the protein. Although there is a little binding of $IsdB^{N2}$ to heme as well, the interaction appears a lot weaker (the band is barely visible in the gel).

2. What does the data in Figure 6 tell you about the requirements for the extraction process using the different constructs in Figure 4?

With the full-length construct ($IsdB^{N1N2}$), there is extraction of heme from metHb. We can also see extraction when two constructs, $IsdB^{N1-L}$ and $IsdB^{N2}$, are mixed together. Thus this combination of constructs appears to have full activity. The other two experiments (c,d) do not show significant extraction of heme. These results indicate that the linker region, N1 and N2 are all important for heme extraction, and that the linker needs to connected to the N1 domain for activity (in experiment d, for example one sees no extraction). We also see that extraction is complete within 1 minute, which gives us a sense of the kinetics of the process. Of course since the reactions are bimolecular the concentrations of the species and the K_ds for their interaction under cellular conditions is key to establishing the kinetic competence.

3. To carry out the experiments described in Figure 7 the Soret band for heme must be distinct when bound to each protein. You can assume that the appropriate controls were carried out on pure proteins to establish that this is the case. Why was the experiment described in Figure 7 carried out and what did it teach the investigators about the function of IsdB?

The data in Figure 7 tells us that $IsdA^{N1}$ can extract heme from metHb as well, but that the process is a lot slower than extraction by $IsdB^{N1N2}$, taking around 12 minutes to go to completion. Figure 7b tells us about the effect of the substrate concentration on the rate of heme extraction. These results, combined with the information given in the question, indicate that $IsdB^{N1N2}$ might extract heme and then transfer the heme to IsdA. Some more experiments would be necessary to study this, including using the full length IsdA to see if there is a difference in heme extraction rate.

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