5.08J Biological Chemistry II (2016)
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.

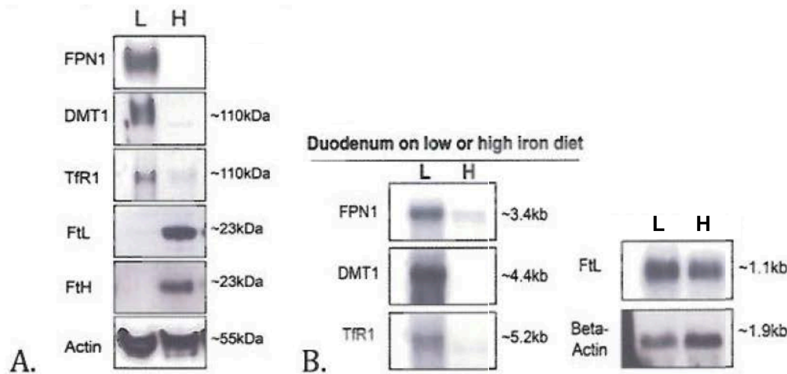

Figure 1A and 1B. **Expression of proteins (A) and mRNA (B)** associated with iron homeostasis in the duodenum (enterocytes, cells involved in iron uptake from the diet) of mice maintained on a low iron diet (L) or a high iron diet (H). **In panel A**, western blots reveal the levels of FPN1 (ferroportin), 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.

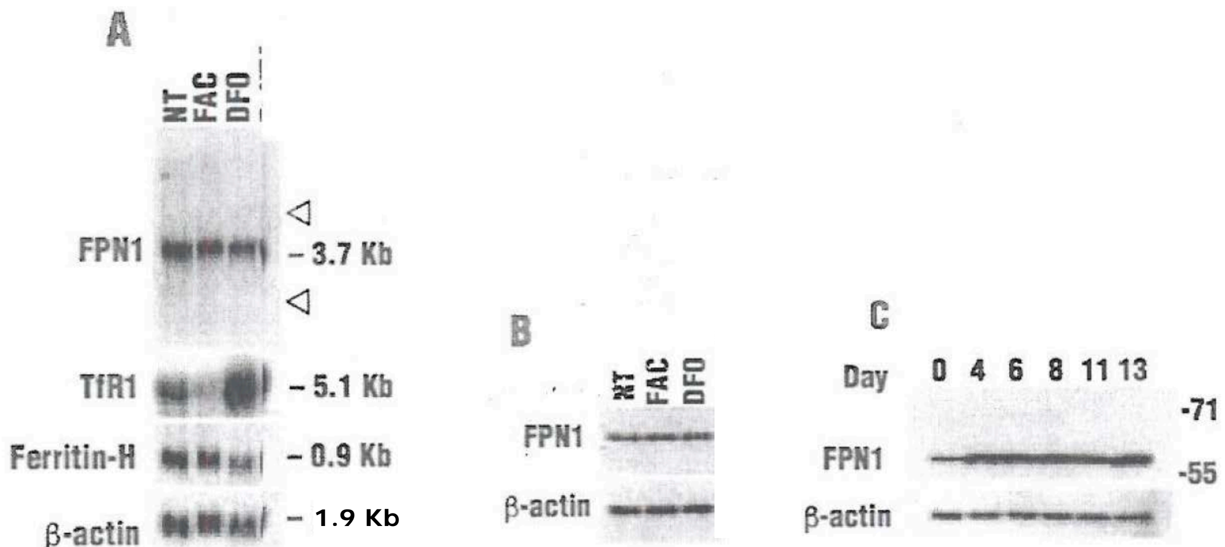

Figure 1 and Figure 2 © source unknown. All rights reserved. This content is excluded from our Creative Commons license. For more information, see https://ocw.mit.edu/help/faq-fair-use.

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.

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.

4. Rationalize the differences between the results reported in Figure 1 and in Figure 2 given the physiological function the cell types.

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).

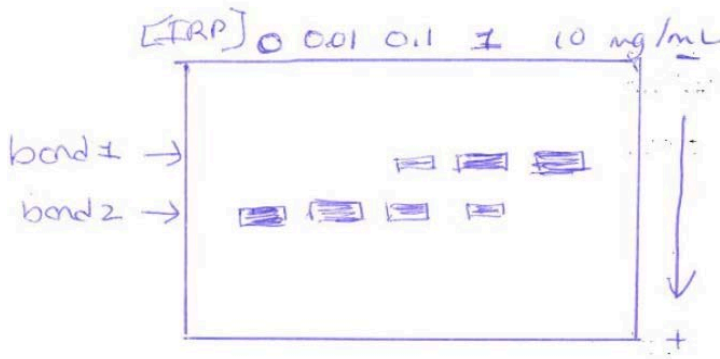

Figure 3. The phosphorimage data (detection of ^{32}P) of the agarose gel. The amount of the IRP is shown at the top of the gel in mg/mL.

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?
2. What is the difference between counts per min (cpm) and disintegrations per min (dpm)?
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 $[IRP1•[IRE]_{bound}$. 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.
4. What control experiments are required to give you more confidence in your analysis?

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.

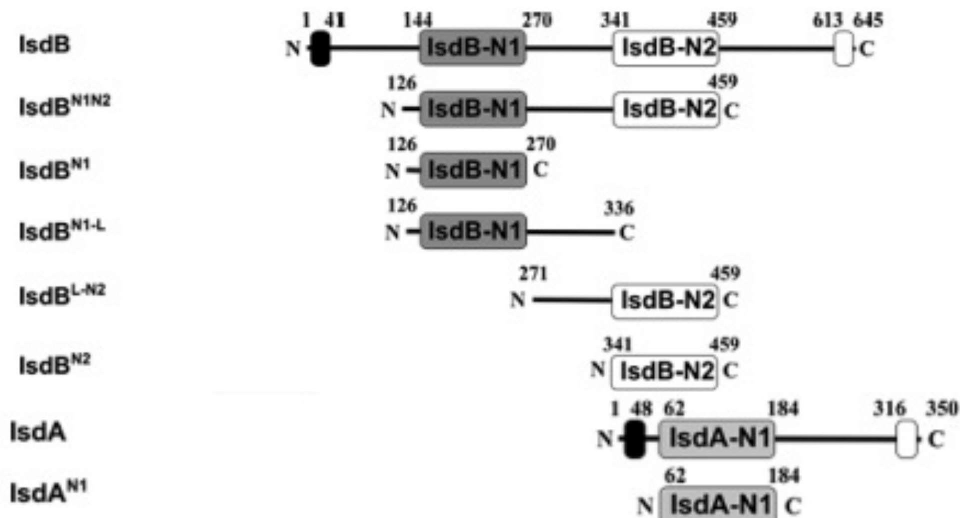

Figure 3. Sequence features of full length IsdB and the recombinant constructs containing the N1, N2 or N1 and N2 NEAT domains that were studied in the experiments described in Figures 5 through 7.

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 4.

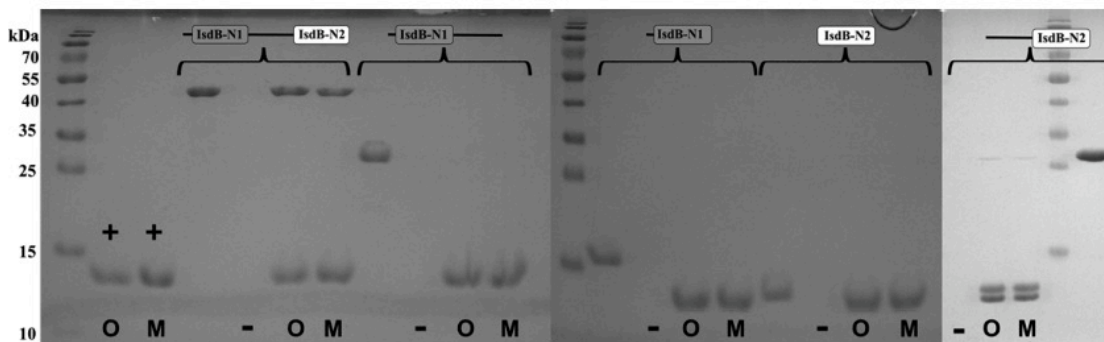

Figure 5. Hb pull-down assay of IsdB constructs. OxyHb (O) and metHb (M) at 20 μM was immobilized on nickel beads, followed by incubation with 20 μM of the distinct IsbB constructs (labels at the top). The bound protein was eluted with 500 mM imdidazole. The positive control (+) shows Hb binding and 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 3 and Figure 5 © American Chemical Society. Bowden, C.F.M., et al. "Hemoglobin Binding and Catalytic Heme Extraction by IsdB Near Iron Transporter Domains." *Biochemistry*, 2014, 53 (14), pp 2286–2294. All rights reserved. This content is excluded from our Creative Commons license. For more information, see https://ocw.mit.edu/help/faq-fair-use.

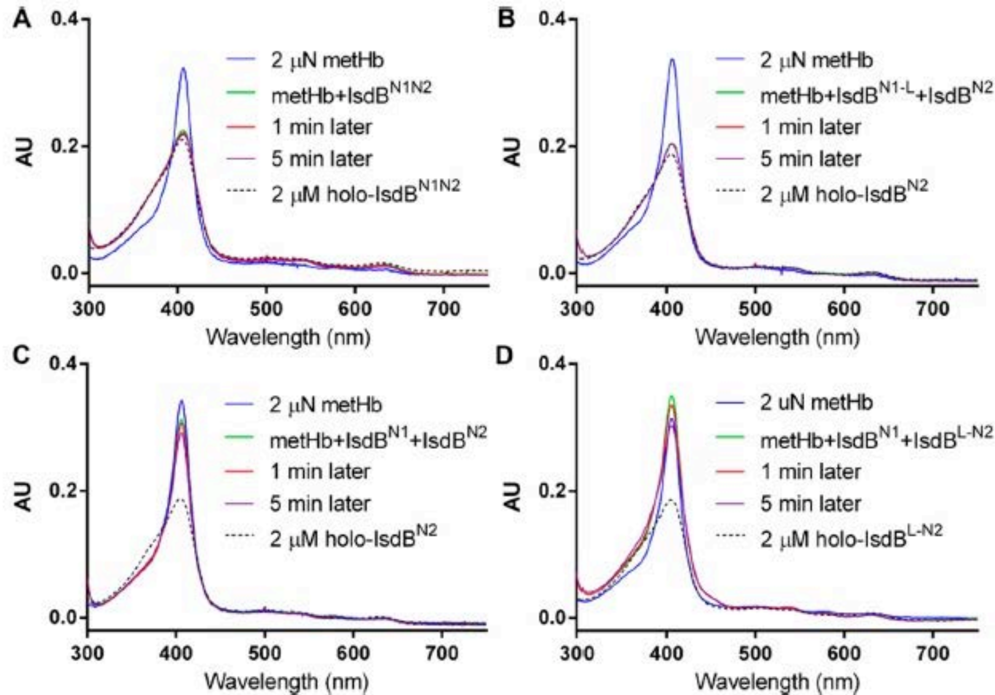

Figure 6. Vis absorption spectra of 2 μM met-Hb mixed with 20 μM of the different IsdB constructs shown in Figure 4.

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

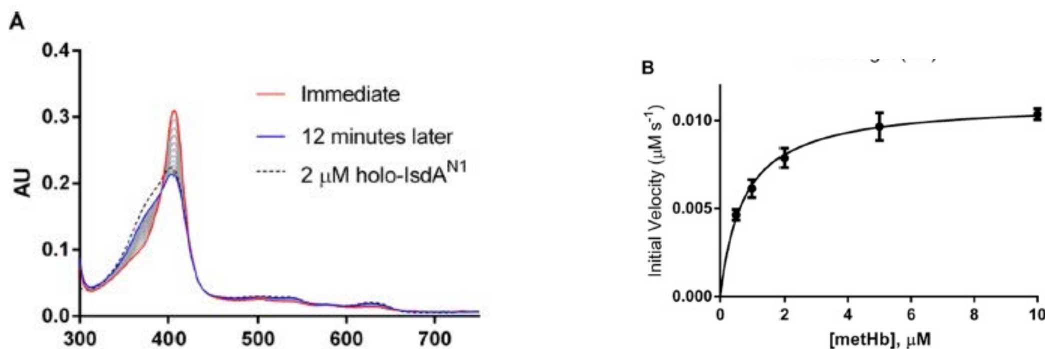

Figure 7. A. Spectral changes observed on incubation of 2 μM met Hb, 50 μM IsdA and 50 nM IsdB. Data for heme reconstituted $IsdA^{N1}$, diluted to 2 μM B by heme concentration - - - are shown for comparison. B. The experiments in A were repeated except with different concentrations of metHb (1 to 10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

Figure 6 and Figure 7 © American Chemical Society. Bowden, C.F.M., et al. "Hemoglobin Binding and Catalytic Heme Extraction by IsdB Near Iron Transporter Domains." *Biochemistry*, 2014, 53 (14), pp 2286–2294. All rights reserved. This content is excluded from our Creative Commons license. For more information, see https://ocw.mit.edu/help/faq-fair-use.

Questions:

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

MIT OpenCourseWare
https://ocw.mit.edu

5.08J Biological Chemistry II
Spring 2016

For information about citing these materials or our Terms of Use, visit: https://ocw.mit.edu/terms