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Analysis of Heme-Reconstitution of Apoenzymes by Means of Surface Plasmon Resonance

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Supporting Information

Other methods for the analysis of apo-enzyme reconstitution

Previously published work on the analysis of apoenzyme reconstitution focused on investigation of heme association and dissociation constants by spectroscopic techniques. For instance, La Mar and co workers estimated the rates of heme loss from mammalian myoglobins by following the NMR changes associated with the loss of heme orientational disorder in newly reconstituted holoprotein.[1] However, this method is generally limited to small proteins which possess high affinity for heme, such as myoglobin. Additionally, rates of heme dissociation from donor enzymes such as horseradish peroxidase or cytochrome c peroxidase and association to apomyoglobin were obtained by monitoring the changes in Soret band. Since for most heme enzymes the spectral changes of the Soret bands are rather small, this type of assay is inconvenient for measuring rates of heme association or dissociation. To circumvent this problem, Hargrove et al constructed a His64-Tyr mutant apoMb, which revealed abnormal spectral properties and high affinity for heme. The use of this mutant as a heme-acceptor enabled determination of the dissociation from various myoglobins and hemoglobin.[2] However, this method is limited to the mutant and it requires significant amounts sample materials.

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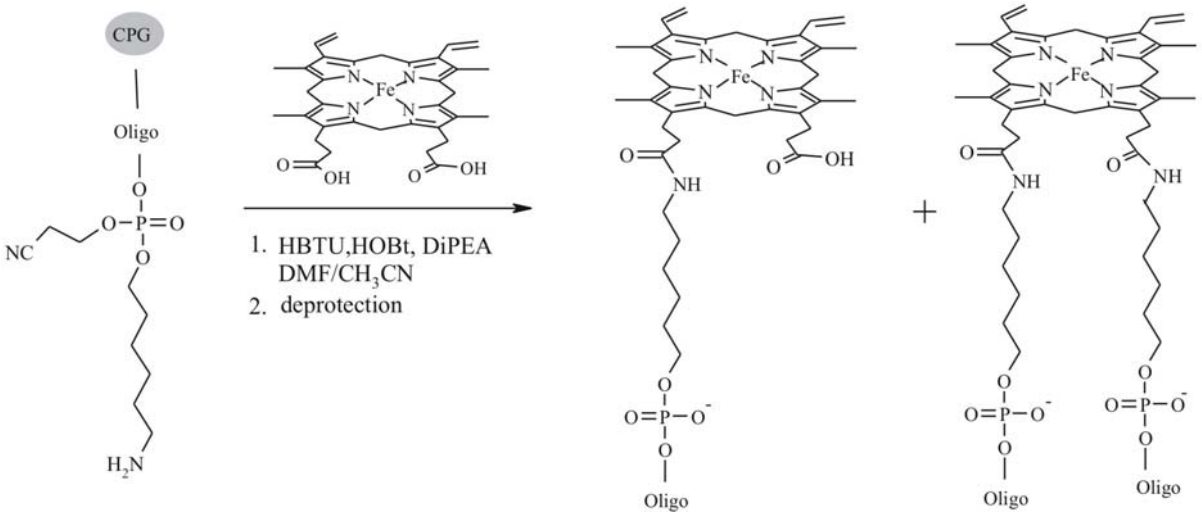
Experimental

Chemicals: Horseradish peroxidase (HRP; purity number of 3), horse heart myoglobin (Mb), 1-ethyl -3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N-hydroxylsuccinimide (NHS) and ethanolamine were purchased from Sigma, neutravidin from Pierce and tert-Octylphenoxy poly(oxyethylene) ethanol (IGEPAL) from Fluka. BIAcore Sensor chips CM5 were obtained from GE Healthcare Europe GmbH (Freiburg, Germany).
5'-biotin modified olionucleotide bcA24 (5'-biotin- AGC GGA TAA CAA TTT CAC ACA GGA) was obtained from ThermoElectron (Ulm, Germany). 5' Amino modified oligonucleotide A24 (5' amino- TCC TGT GTG AAA TTG TTA TCC GCT) for hemeA24 synthesis was purchased as unprotected and bound on solid support form Tib-Molbiol (Berlin, Germany). HemeA24 was synthesized using previously described procedure.[3]

BIA Measuraments. Biospecific interaction analysis (BIA) was performed using a BIAcore instrument 3000 from Pharmacia Biosensor AB (Uppsala, Sweden).
Immobilisation of capture oligomer and hybridization of heme oligomer. Degassed 20 mM HEPES buffer (20 mM HEPES; 150 mM NaCl, 0.005% Igepal pH 7.4) was used as a running buffer throughout experiments with a continuous flow of buffer at 5 uL/min at 25 °C. Sensor Chip CM5 (BIACORE; Uppsala, Sweden) was coated with neutravidin using EDC/NHS coupling strategy. In brief, 0.1 M aqueous solutions of EDC and NHS were introduced to the chip followed by 100 μg/ml solution of neutravidin in sodimum acetate buffer (pH 6.0) and coupling was allowed for 15 min. Unmodified reactive sites were then blocked with ethanolamine (35 μL 1M ethanolamine hydrochloride, adjusted to pH 8.5 with NaOH). Before the immobilization of biotinyalted capture oligo bcA24, five wash cycles with 10 μL 100mM HCl were performed, followed by a buffer wash. Biotinylated capture oligomer (2 μM in running buffer) was then injected for 8 min over the neutravidin coated chips. Loosely attached material was removed by three cycles of 10 μL of 100 mM NaOH. Heme-modified oligonucleotide (heme-A24) was allowed to hybridise to the capture oligomer by injecting 2 μM solution in running buffer for 15 min, followed by 8 min buffer wash.



Scheme S1: The solid phase synthesis of heme modified oligonucleotide using amide coupling strategy.[3]

Apoenzyme reconstitution. The apoenzymes apoMb and apoHRP, lacking their heme cofactor, were prepared by Teale's 2-butanone method.[4] Briefly, 200uL of a 50uM aqueous solution of the enzyme was acidified to pH 2.3 (Mb) or 2.5 (HRP) using 0.1 M HCl and the heme was extracted by addition of ice cold 2-butanone. The colorless apoenzyme solution was then passed through a NAP 5 size exclusion column to remove the excess of 2-butanon.

Solutions of apoenzymes were prepared in running buffer and used fresh prior to each experiment. For BIA analyses, cycles of heme D25 hybridisation followed by reconstitution with apoenzyme solution were programmed in such way that the concentration of hemeA24 remained constant (2μM) and the concentration of the enzymes varied from 50 to 400 nM. In a typical run, 120 μL of hemeA24 were injected over 24 min, followed by 10 mM HCl and buffer wash (1 min). Subseqeuntly, 120 μL of apoenzyme solution were injected over 480 min. Dissociation of immobilized apoenzyme was affected by injecting running buffer only and it was followed for 30 min. After each reconstitution event, the surface was regenerated with 2 cycles of 100 mM HCl (2 min, each) and 5 cycles of 100 mM NaOH (2 min, each) wash to remove reconstituted Mb as well as unbound hemeA24 and to prrepare the chip for the next hybridization and reconstitution cycle. Binding and dissociation curves were recorded automatically using pre-programmed analytical cycles. The interactions were monitored continuously as the change in SPR signal.

Data analysis was carried out using BIAcore Evaluation 4.1 software (Pharmacia). The pseudo first order kinetic model was used. Series of controls were carried out, which included the injection of apoenzyme over surfaces containing non-heme modified oligomers, injection

of solutions containing holoenyzmes or no enzymes at all (Figure S3). No significant increase in RU was observed for the controls, thus indicating that the binding observed in the kinetic experiments was due exclusively to the reconstitution of apoenzymes. The amounts of bound oligonucleotides and reconstituted enzymes were calculated from RU units (in ng) and then transferred to moles, using molecular mass of the corresponding molecules. The k_{diss} (in s^{-1}) and k_{ass} (in 1/Ms) were obtained from the plot of k_{obs} (obtained from the pseudo first order curve fitting) *vs* concentration of apoenzymes. K_D was then calculated from the k_{diss}/k_{ass} ratio.

Determination of rate constants[5]:

First order kinetic model

Association:

$$A + B \xrightarrow{k_{ass}} AB$$

$$\frac{d[AB]}{dt} = k_{ass}[A]_t \times ([B]_0 - [AB]_t) - k_{diss} \times [AB]_t$$

$[B]_0 = RU_{MAX}$ Free ligand concentration ≈ constant $C = [A]_t$

$$\frac{dRU}{dt} = k_{ass} \times C \times RU_{MAX} - (k_{ass} \times C + k_{diss}) \times RU_t$$

$$k_{obs} = k_{ass} \times C + k_{diss}$$

Dissociation:

$$AB \xrightarrow{k_{diss}} A + B$$

$$\frac{dRU}{dt} = k_{diss} \times RU_t$$

$$\ln(RU_{t1} - RU)_{tn} = k_{diss} \times (t_n - t_1)$$

$$K_D = \frac{k_{diss}}{k_{ass}}$$

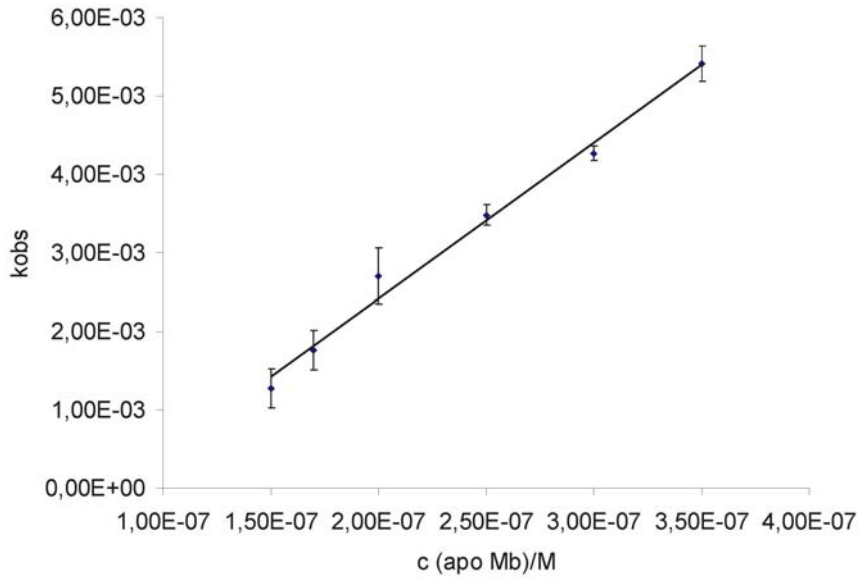


Figure S1: Linear variation of k_{obs} (obtained from curve fitting using Bia evaluation 4.1 software) with concentration of apoenzyme. The slope of the curve corresponds to k_{ass} (in 1/Ms). k_{diss} was obtained from each curve fitting and average mean values were taken. K_D was calculated as k_{diss}/k_{ass} ratio.

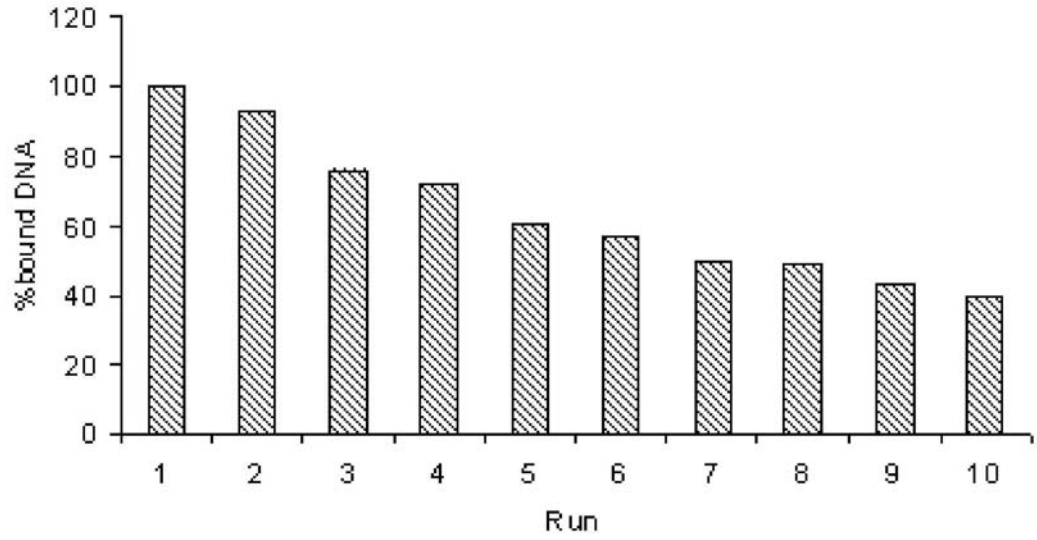


Fig S2: Amount of heme-A24 hybridised with the chip-immobilized bcA24. Note that about 50% of initial hybridization capacity is maintained even after 10 cylces of regeneration.

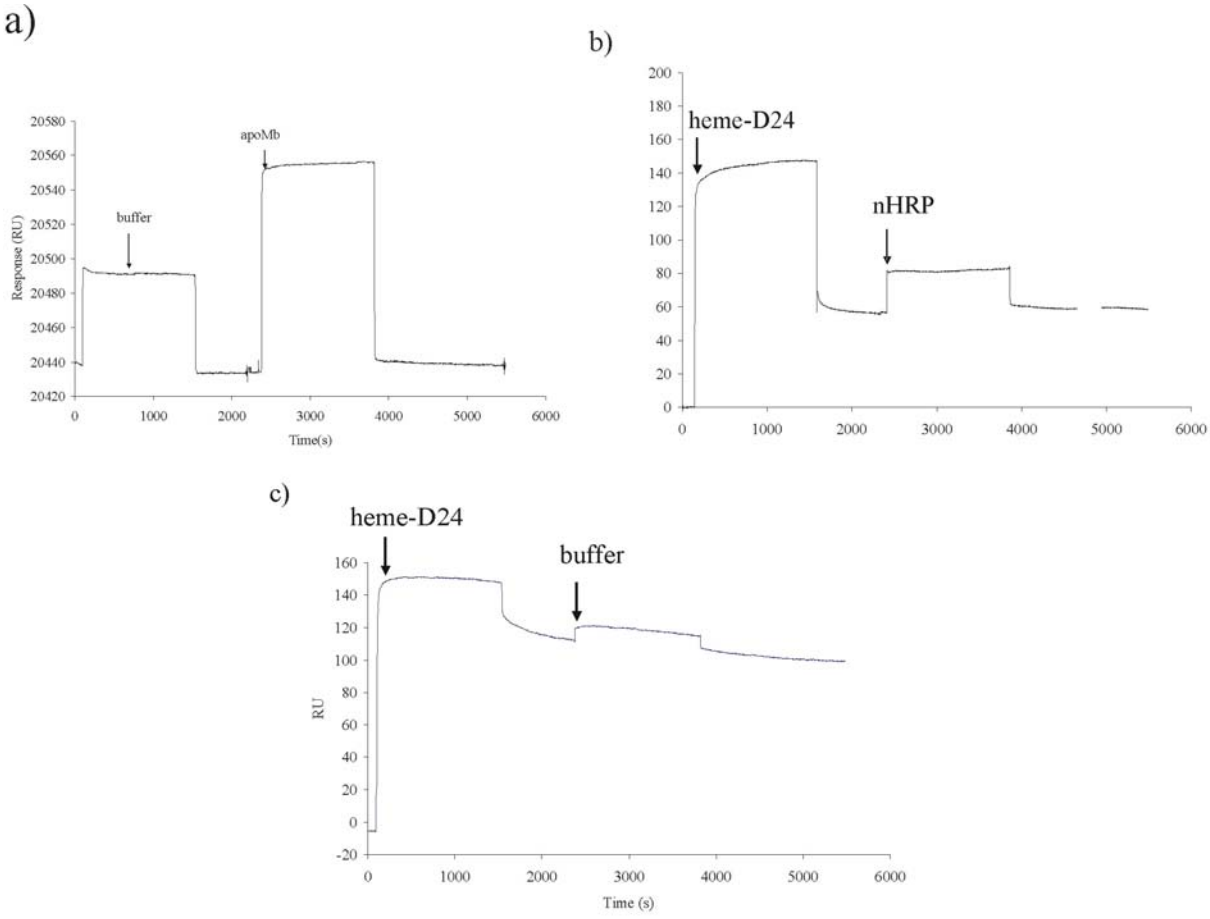
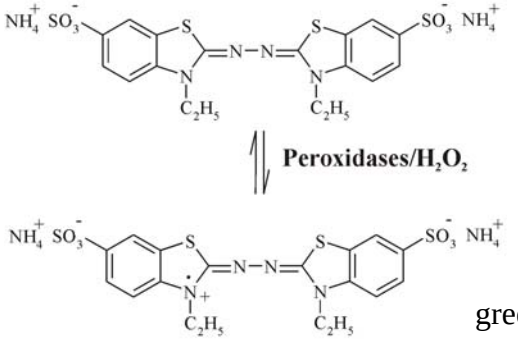


Fig. S3: Control experiments; a) injection of apoMb after no heme-A24 hybridisation (buffer used instead); b) native HRP injection over chip-bound heme-A24; c) no apo enzyme (buffer used).

ABTS activity assay for on-chip reconstituted Mb and HRP

2 ml ABTS/peroxide solution was prepared by mixing ABTS (final concentration of 50μM in phosphate citrate buffer pH 5.5) and H_2O_2 (final concentration of 1mM). A chip containing reconstituted enzymes (after BIA experiments) was immersed in freshly prepared solution, left for 5 min and the absorbance of ABTS radical cation was measured at 405 nm.



green, λ_{MAX}=405 nm

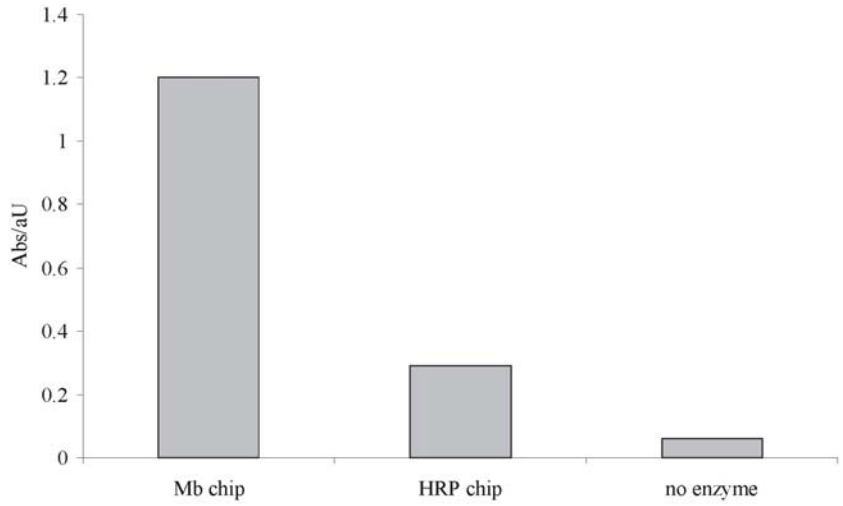


Fig. S4: Absorbance of ABTS radical cation at 405 nm recorded after immersion of enzyme containing chip into the ABTS/H_2O_2 solution.

Determination of rate constant for DNA-dehybridisation:

Separate experiments were performed to measure the dissociation rate constant of heme-A24 using BIA. The values obtained are summarized in Table S5 and they are similar to that of pure DNA, determined in an earlier work.[6]

Table S5: Values of k_{diss} and K_D for dehybridisation of hemeA24 conjugate.

	$10^{-4}k_{diss}(s^{-1})$	$10^{-8}K_D(M)$
hemeA24	0.16±0.03	0.10

References

[1] G. N. La Mar, H. Toi, R. Krishnamoorthi, *J. Am. Chem. Soc.* **1984**, *106*, 6395-6401.
[2] M. S. Hargrove, E. W. Singleton, M. L. Quillin, L. A. Ortiz, G. N. Phillips, Jr., J. S. Olson, A. J. Mathews, *J Biol Chem* **1994**, *269*, 4207-4214.
[3] L. Fruk, C. M. Niemeyer, *Angew Chem Int Ed Engl* **2005**, *44*, 2603-2606.
[4] F. W. Teale, *Biochim. Biophys. Acta* **1959**, *35*, 543.
[5] I. Chaiken, S. Rose, R. Karlsson, *Anal Biochem* **1992**, *201*, 197-210.
[6] C. M. Niemeyer, W. Burger, R. M. Hoedemakers, *Bioconjug Chem* **1998**, *9*, 168-175.