Mutant hemoglobin stability depends upon location and nature of single point mutation

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The temperature dependence of the rates of heme release from the β subunits of methemoglobin A and 5 β mutant methemoglobins has been determined. The rates were largest for two hemoglobins with mutations distal to heme, previously known to be unstable. The other 3 mutants also released heme faster than A. These hemoglobins, with single point mutations at the $\alpha_1\beta_2$ interface, were previously thought to be stable. The low reported yields of the 5 mutant proteins covaries with the relative rates of heme release from the met species.

Hemoglobin *Point mutation* *Heme release* *Protein dynamics* *Oncogene*

1. INTRODUCTION

Human hemoglobin variants exhibit a wide range of stabilities in the red cell [1]. Point mutations at positions remote from the heme 'pocket' or from the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ interfaces do not markedly influence the stability of the mutant protein [2]. However, mutations neighboring the heme result in anemia and increased Heinz body formation [3]. The mechanism(s) producing these conditions are not fully identified but must be mutation dependent. Mutations decrease the negative charge upon the β tetramer and inhibit the formation of functional $\alpha\beta$ dimers, producing mild anemia [4]. Alternatively the mutant β tetramer may undergo oxidation to irreversible forms of hemichromes which give Heinz bodies [5]. Another proposal for Heinz body formation and anemia is a rapid loss of heme from the mutant subunit followed by precipitation of the apoprotein [3]. The latter hypothesis has only been invoked for a few mutant hemoglobins with mutations neighboring heme [3].

Abbreviations: Hb, hemoglobin; Kö, Köln; HS, Hammersmith; SM, Saint Mandé; HD, Hôtel Dieu; Mö, Malmö

We have investigated the rate of heme release from normal metHb A and from 5 mutant methemoglobins. Köln (β98 val → met) and Hammersmith (β42 phe → ser) with mutations neighboring the β heme are classic unstable hemoglobins. Saint Mandé (β102 asn → tyr), Hôtel Dieu (β99 asp → gly), and Malmö (β97 his → gln) are mutated at the $\alpha_1\beta_2$ interface but are not considered unstable hemoglobins. Here we have followed the release of heme by monitoring spectrophotometrically the transfer of heme to apomyoglobin. We find that hemoglobins SM and HD are mildly unstable. The low reported yields of these proteins may be due to enhanced loss of the β heme. We also find the Arrhenius activation energies may be correlated with position and nature of the point mutation.

2. MATERIALS AND METHODS

Hemoglobins HS, Mö, SM and HD were purified from the hemolyzates of heterozygous donors by chromatography on CM-52 (Whatman) using a pH gradient [6]. Hemoglobin Kö was isolated by the above method from the hemolyzate of a donor with β thalassemia. Only those fractions of HS and Kö with near normal A_{280}/A_{415} ratios (~0.26) were

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used to avoid contamination with globin. The purified proteins were concentrated by ultrafiltration (YM-5 membrane, Amicon) under nitrogen, dialysed against 50 mM potassium phosphate, 1 mM EDTA (pH 7.0) and stored as beads in liquid nitrogen. The spontaneous heme release was followed for 16 h at 409 nm using apomyoglobin as the heme scavenger [7]. Measurements were done in the above buffer, with 2-fold excess per heme basis of potassium ferricyanide, added 30 min before initiating the reaction by adding about 3-fold excess apomyoglobin. Rate constants were estimated on an ABC 80 computer using a non-linear least-squares curve-fitting procedure with linear combination of two simultaneous first-order rate terms. From 30 to 50 data points were used for each calculation.

A Beckman DU-7® spectrophotometer with a jacketed cell holder was thermostatted with an LKB Julabo VL water bath. Sample temperatures were determined by a Cormack thermistor with a K76P/1 thermocouple.

3. RESULTS

The spontaneous release of heme was observed to be biphasic, consistent with previous results on exchange of heme between unlike hemoglobins [8]. The rapid phase was usually complete within 2–3 h while the slower phase exhibited a half-time of about 10 h. The rapid phase corresponds to the release of heme from the β subunits and the slower from the α subunits [8]. Each phase comprised approximately half of the total absorbance change in good cases; however, release of heme from the α subunits at higher temperatures was accompanied by a conspicuous globin precipitation. This appeared spectrophotometrically as dramatically increased rate of absorbance change following the slow absorbance change of α heme transfer. Because of this complication the rate constants for heme release from the α subunits are thought to be untrustworthy.

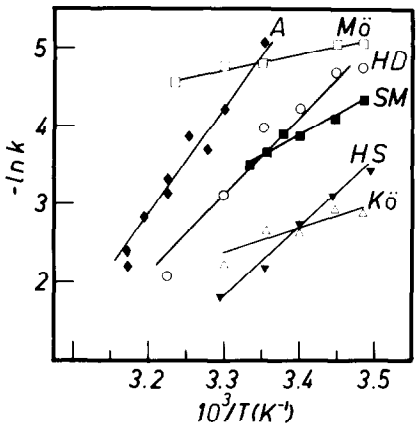

Fig.1. The Arrhenius plots of heme release from methemoglobins A and 5 mutant methemoglobins. Activation energies are given in table 1.

Fig.1 shows Arrhenius plots of heme release from the various methemoglobins. All mutants rapidly precipitated above 37°C, so activation energies are calculated on data from a restricted temperature range. Error bars have been omitted because the computed errors are smaller than the symbols. Large differences in heme release rates and activation energies are apparent from this plot.

Table 1

Energies of β heme release and preparative yields of normal and mutant hemoglobins[a]

Hemoglobin	Mutation	[b]$cm^3 \cdot M^{-1}$	$\log A$ (min^{-1})	E_a (kJ/mol)	k_1 (min^{-1})[c]	Yield (%)	
A	–	–	19.3	124 ± 1	0.0064	50	
Malmö	β97 his → gln	−4.7	1	17.4 ± 0.3	0.0082	48	[6]
Hôtel Dieu	β99 asp → gly	−36.5	14.5	92 ± 2	0.018	43	[9]
Saint Mandé	β102 asn → tyr	+30.9		43.6 ± 5	0.026	38.5	[10]
Köln	β98 val → met	+10.8	4	29 ± 0.8	0.070	~25	[11]
Hammersmith	β42 phe → ser	−40.3	11.3	70 ± 0.6	0.114	40	[12]

[a] In 50 mM phosphate, 1 mM EDTA (pH 7.0)
[b] Change in van der Waals volumes; from [19]
[c] At 25°C

The kinetic parameters in table 1 show that all mutants exhibit lowered Arrhenius energies. The absolute rate of heme release at 25°C is higher for any mutant than for A. The only mutant which appears more stable than metHb A is metMö at temperatures above 25°C. The heme release from metSM was measured at several different concentrations between 2.5 and 8.4 μM heme at 21°C, and the rate constant was found to be independent of donor concentration and dimer-tetramer equilibrium position.

4. DISCUSSION

Using apoMb as a heme 'trap' we have measured the rate of heme release from horseradish peroxidases A and C, cytochrome *c* peroxidase and legHb [7]. However, measurement of the release from metHb A allows, for the first time, comparison of our results with others. In a study employing ^{59}Fe labelled heme, authors in [8] found that heme exchange occurred more rapidly between β and γ, as we have here deduced. Their reported rate constants and activation energy (85 kJ/mol) are compatible with our rates and activation energy (124 kJ/mol). Complete agreement is not to be expected since they measured exchange rates and not heme release.

The high heme release rates and low activation energies were expected for HS and Kö. Both mutations occur in polypeptide segments forming the heme pocket, and any disturbance of the precise fit between heme and protein should alter the heme binding constant. The increased rates and lowered activation energies for the other 3 mutants were unexpected. While the β102 residue does contact the heme pyrrole II (Fischer convention [13]) these mutants (HD, SM, Mö) have been previously thought of as being stable. We have reported low yields for SM [10] and HD [9]. For HD and Mö neither mutation occurs at positions neighboring heme but at the $\alpha_1\beta_2$ interface. One possible explanation for their instability is that mutations at the β99 and β97 positions are transmitted through the F helix to val 98, which contacts pyrrole II, disrupting packing between heme and protein and increasing rates of heme release. Another possibility is that any mutation at the $\alpha_1\beta_2$ region increases the flexibility of the polypeptide and lowers the activation energy for heme release. From our initial study it is difficult to decide between these possible mechanisms. However, table 1 shows that activation energies are lowered more by mutations which increase side chain van der Waals volume.

The importance of the study of single point mutant proteins has recently been highlighted by the finding that the normal allele H-*ras*-1, which codes for the mammalian protein p21, becomes an oncogene by mutation of a single base pair [14]. The amino acid normally coded is glycine, and mutation to glutamic acid or arginine results in the oncogenic proteins NMU-H-*ras* and v-*ras*, respectively. The amino acid sequence of p21 is very hydrophobic in the neighborhood of the mutatable glycine, and this polypeptide sequence may reside in the interior of p21. This situation is similar to that of hemoglobin Riverdale-Bronx (β24 gly → arg) [15]. The β24 glycine plays an important role in the internal structure of normal hemoglobin, where sliding of the E helix past this residue is demanded by 'cooperativity'. Hemoglobin Riverdale-Bronx is considered an unstable protein due to increased van der Waals volume of the β24 arginyl residue which destabilizes the B and E helixes. If p21 is a regulatory protein, or a subunit of a regulatory protein as suggested [17,18], then mutation of an internal glycyl residue to any other residue would be expected to alter the regulatory properties and certainly the stability of the p21 related oncogenic proteins. It would be of interest to study the thermal stabilities of NMU-H-*ras* and v-*ras* oncogenic proteins.

Table 1 also shows that the yields of mutant hemoglobins covary with heme release rates. Previous studies used heat denaturation to assay hemoglobin stability, a test which combines autooxidation with heme release processes. As a check of the relative importance of autooxidation and heme release we assayed the oxidation of SM and A by the azide test of authors in [18] and found their oxidation rates to be the same (unpublished). Therefore, low yield of this protein is probably due to facile heme release.

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REFERENCES

[1] Labie, D. and Wajcman, H. (1974) Haematologia 8, 87–95.

[2] Pettigrew, D.W., Roméo, P.H., Tsapis, A., Thillet, J., Smith, M.L., Turner, B.W. and Ackers, G.K. (1982) Proc. Natl. Acad. Sci. USA 79, 1849–1853.

[3] Jacob, H.S. and Winterhalter, K.H. (1970) J. Clin. Invest. 49, 2008–2016.

[4] Bunn, H.F. and McDonald, M.J. (1983) Nature 306, 498–500.

[5] Rachmilewitz, E.A., Peisach, J. and Blumberg, W.E. (1971) J. Biol. Chem. 246, 3356–3366.

[6] Zak, S.J., Geller, G.R., Krivit, W., Tukey, D., Brineholt, B., Jones, R.T., Bunn, A.F. and McCormack, M. (1976) Br. J. Haematol. 33, 101–104.

[7] Smith, M.L., Ohlsson, P.I. and Paul, K.G. (1982) in: Cytochrome P-450, Biochemistry, Biophysics and Environmental Implications (Hietanen, E. et al. eds) pp. 601–606, Elsevier, Amsterdam, New York.

[8] Bunn, H.F. and Jandl, J.H. (1966) Proc. Natl. Acad. Sci. USA 56, 974–978.

[9] Blouquit, Y., Braconnier, F., Galacteros, F., Arous, N., Soria, J., Zittoun, R. and Rosa, J. (1981) Hemoglobin 5, 19–31.

[10] Arous, N., Braconnier, F., Thillet, J., Blouquit, Y., Galacteros, F., Chevrier, M., Bordahandy, C. and Rosa, Y. (1981) FEBS Lett. 126, 114–116.

[11] Carrell, R.W., Lehmann, H. and Hutchinson, H.E. (1966) Nature 210, 915–916.

[12] Dacie, J.V., Shinton, N.K., Gaffney, P.J., Carrell, R.W. and Lehmann, H. (1967) Nature 216, 633–635.

[13] Fischer, H. and Orth, H. (1968) Die Chemie des Pyrroles, Johnson Reprint, New York.

[14] Sukumar, S., Notario, V., Martin-Zanca, D. and Barbacid, M. (1983) Nature 306, 658–661.

[15] Ranney, H.M., Jacobs, A.S., Udem, L. and Zalusky, R. (1968) Biochem. Biophys. Res. Commun. 33, 1004–1011.

[16] Gallwitz, D., Donath, C. and Sander, C. (1983) Nature 306, 704–707.

[17] De Feo-Jones, D., Scolnick, E.M., Koller, R. and Dhar, R. Nature 306, 707–709.

[18] Wallace, W.J., Houtchens, R.A., Maxwell, J.C. and Caughey, W.S. (1982) J. Biol. Chem. 257, 4966–4977.

[19] Bondi, A., (1964) J. Phys. Chem. 68, 441–451.