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## Note

# ATP–hemoglobin: anomalous oxygen binding properties*

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ATP-modified hemoglobin (ATP–Hb) has recently been investigated by Greenburg and Maffuid [1] as a potential hemoglobin-based blood substitute. Their results show that, relative to pyridoxal 5′-phosphate-labelled hemoglobin (PLP–Hb), ATP–Hb has a longer plasma half-life and, as the result of a larger reduction in oxygen affinity, it may have superior oxygen delivery characteristics. Consequently, ATP–Hb has considerable potential as a blood substitute. Scannon [2], however, has reported that labelling Hb with ATP results in no significant decrease in oxygen affinity. The latter results contrast those of Hsia et al. [3], who have recently optimized Greenburg and Maffuid's preparative procedures and have reported that, when stripped of unlabelled Hb by affinity chromatography, ATP–Hb has a markedly reduced oxygen affinity. Critical assessment of the potential of ATP–Hb as a blood substitute requires the clarification of this discrepancy. In the present communication, we report the high-performance liquid chromatographic (HPLC) resolution and partial characterization of ATP–Hb prepared by the method of Scannon [2]. The results indicate that major modified components contain only traces of phosphate and show no significant reduction in oxygen affinity. Furthermore, a minor component, $(ATP)_2Hb$, has been isolated and found to show a marked reduction in oxygen affinity. The impact of these observations on the potential of ATP–Hb as a hemoglobin-based blood substitute is discussed.

## EXPERIMENTAL

### *Chemicals and reagents*

Unless otherwise stated, all reagents and buffers were purchased from Sigma (St. Louis, MO, U.S.A.) or BDH Chemicals (Toronto, Canada). Stroma-free

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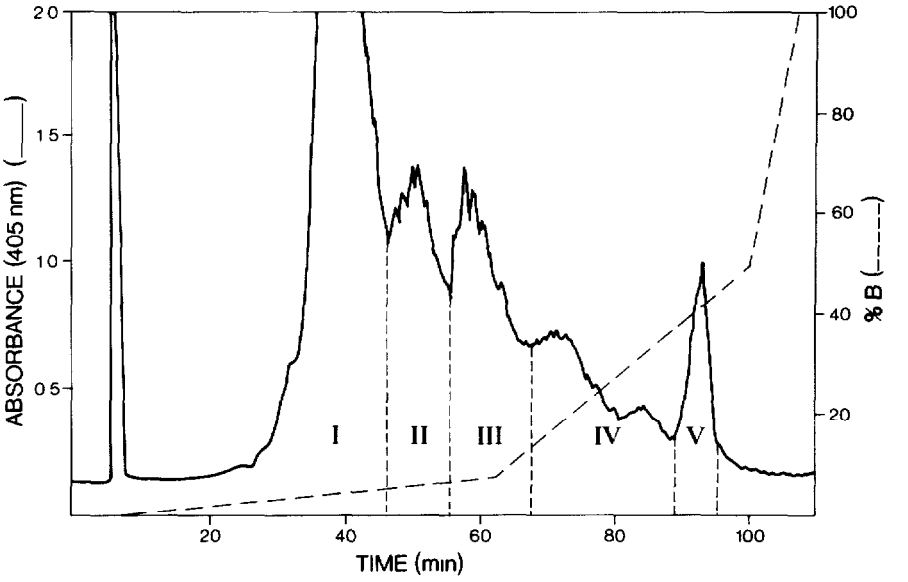


Fig. 1. Preparative anion-exchange HPLC profile of the ATP–Hb reaction mixture (200 mg) eluted from a Pharmacia Mono Q HR 16/10 column at a flow-rate of 7.0 ml/min. Buffer A is 20 m*M* Tris–HCl, pH 8.0 and buffer B is 0.5 *M* sodium chloride in buffer A. Fractions I–V were collected as indicated.

hemoglobin (SFH) was prepared from outdated whole blood (Canadian Red Cross) as described [4]. UV–VIS spectra were obtained with a Beckman DU 50 spectrophotometer (Beckman, CA, U.S.A.).

ATP–Hb was prepared using a method similar to that described by Scannon [2]. A mixture of 2-octanol (20 $\mu$l) and SFH (5.0 $\mu$mol) in 50 m*M* Tris–HCl buffer pH 7.5 (20 ml) was injected into a 50-ml vial equipped with a magnetic stirring bar, a gas inlet and a gas outlet. After wet nitrogen was bubbled through the stirred 4°C solution overnight to deoxygenate the SFH, periodate oxidized ATP (o-ATP, 15 $\mu$mol) in deoxygenated distilled water (100 $\mu$l) was added. The mixture was allowed to react for 6 h at 4°C and then was quenched by dialysis with pH 7.5, 50 m*M* Tris–HCl (3×1 l).

*Procedure*

The preparative anion-exchange HPLC separations were carried out with a Pharmacia Mono Q HR 16/10 column using the Pharmacia fast protein liquid chromatographic (FPLC) system (Pharmacia Canada) previously described [5]. Buffer A was 20 m*M* Tris–HCl pH 8.0 and buffer B was buffer A plus 0.5 *M* sodium chloride. Typically, the ATP–Hb mixture (200 mg) in buffer A (5 ml) was injected via a super loop and then the chromatograph was developed at a flow-rate of 7 ml/min using the gradient shown in Fig. 1. The effluent was collected in 14-ml fractions which were pooled as indicated in Fig. 1.

The pooled ATP–Hb fractions were desalted, concentrated to approximately 5 g/dl with an Amicon ultrafiltration cell equipped with a PM 10 (10 000 molecular weight cutoff) membrane. The specimens were then dialyzed against 50 m*M*

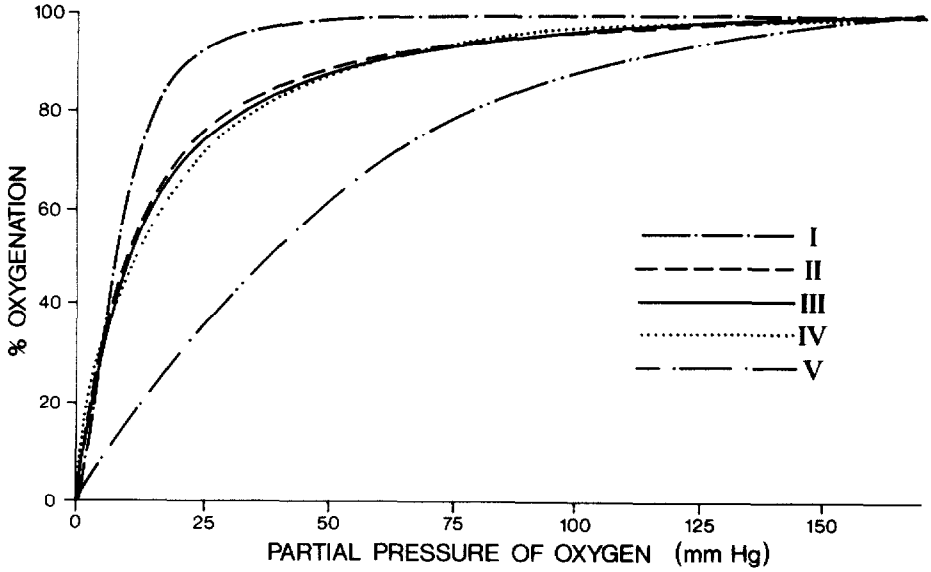


Fig. 2. Oxygen dissociation curves of fractions I–V of the ATP–Hb reaction mixture obtained with an Aminco Hom-O-Scan oxygen dissociation analyzer. The samples were analyzed at 37 °C in 50 m*M* Bis–Tris, pH 7.0, with hemoglobin concentrations of 3.5 g/dl.

Bis–Tris pH 7.0 and then diluted to a uniform concentration of 3.5% with the same buffer. The oxygen dissociation curves shown in Fig. 2 were obtained with an Aminco Hemo-O-Scan oxygen dissociation analyzer. Values for the partial pressure of oxygen at 50% saturation ($P_{50}$) were read directly from the curves.

Aliquots of the ATP–Hb fractions were analyzed in triplicate for total phosphate using the method of Ames and Dubin [6] with the only modification to the procedure being a four-fold increase in the amount of magnesium nitrate hexahydrate used in the ashing phase of the analysis.

The reversed-phase HPLC separations were performed with a Pharmacia Pro RPC HR 10/5 column installed in a Waters HPLC system (Waters Canada) consisting of a 680 automated gradient controller, two 610 pumps, a 480 Lamda Max LC detector and a Hewlett-Packard 3390A integrator (Hewlett-Packard Canada) using a method similar to that described by Jeppson et al. [7]. The samples (10 $\mu$g) in the initial buffer mixture (100 $\mu$l) were eluted with the gradient of buffer A (39% acetonitrile–water containing 0.3% trifluoroacetic acid) and buffer B (52% acetonitrile–water) shown in Fig. 3 at a flow-rate of 0.2 ml/min.

## RESULTS

### *Anion-exchange HPLC separation of ATP–Hb*

After o-ATP-modified Hb (o-ATP–Hb) was prepared by the method of Scannon [2], it was necessary to resolve the mixture. We have recently reported high-resolution separations of PLP–Hb using anion-exchange HPLC [5]. Briefly, these separations were performed with a quaternary ammonium monobead support (Pharmacia Mono Q) under weakly basic elution conditions (pH 8.0) which

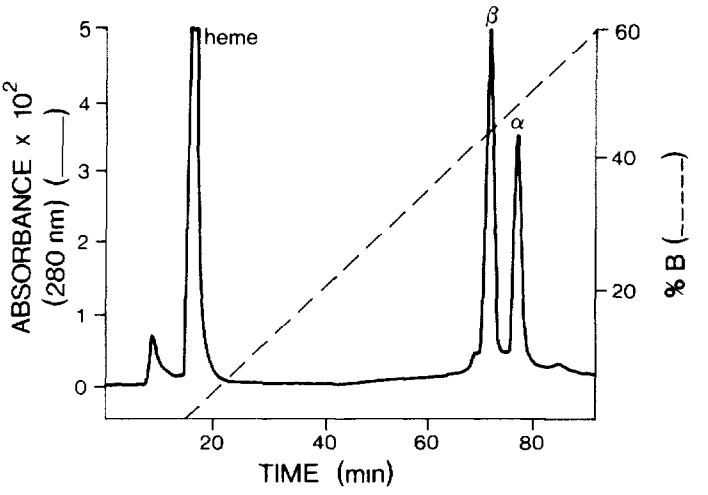


Fig. 3. Analytical reversed-phase HPLC profile of fraction I of the ATP–Hb reaction mixture (10 $\mu$g) eluted from a Pharmacia Pro RPC 5/10 column at a flow-rate of 0.2 ml/min. Buffer A is 39% aqueous acetonitrile plus 0.3% trifluoroacetic acid and buffer B is 52% aqueous acetonitrile.

minimize complications resulting from autoxidative formation of the corresponding methemoglobin derivatives. Moreover, the separations were found to be rapid, highly reproducible and easily scaled up to a preparative level. This methodology was, therefore, applied to the resolution of o-ATP/Hb reaction mixtures.

Fig. 1 shows a typical chromatogram obtained from 200 mg of the o-ATP/Hb reaction mixture and indicates the presence of at least five components. Fraction I has the same retention time as does the major component of SFH while the modified products all show decreased cationic mobility. The use of a shallower gradient failed to improve the resolution. Accordingly, the fractions were pooled as indicated in Fig. 1.

*UV–VIS spectra*

UV–VIS spectra obtained from each of the components were indistinguishable from that of SFH. Therefore, significant autoxidative formation of methemoglobin derivates did not occur during the anion-exchange HPLC separation [8].

*Phosphate analyses*

The results of the moles phosphate to moles Hb analyses for each of the fraction are listed in Table I. The first four fractions contain only traces of phosphate; far less than the 3 moles phosphate per mole Hb required of a 1:1 ATP–Hb adduct. Fraction V, however, contains approximately 6 moles of phosphate per mole of Hb and, therefore, represents an Hb derivative which bears two molecules of o-ATP.

*Oxygen dissociation curves*

The oxygen dissociation curves obtained from each of the fractions are shown in Fig. 2 while the $P_{50}$ values are listed in Table I. As anticipated, fractions II, III

TABLE I

PHYSICAL CHEMICAL PROPERTIES OF THE FRACTIONS OF ATP-Hb

| Fraction | Moles phosphate/moles Hb | $P_{50}$ (mmHg) |
|---|---|---|
| I | 0.1 (±0.2) | 8.0 (±0.5) |
| II | 0.1 | 10.5 |
| III | 0.2 | 11.0 |
| IV | 0.8 | 12.0 |
| V | 5.9 | 38.0 |

and IV show little change in $P_{50}$ from that of fraction I whereas a large reduction in oxygen affinity is evident in the curve obtained from fraction V ($P_{50}$=38 mmHg).

*Reversed-phase HPLC analysis*

The fractions were separated into their $\alpha$, $\beta$ and modified chains by reversed-phase HPLC using the same type of column (Pharmacia Pro RPC HR 10/5; $C_1$–$C_8$, 300 Å pore size, 5-$\mu$m beads) and elution conditions as those used by Jeppson et al. [7] in their characterization of Hb-Linköping, a human variant. The chromatogram obtained from fraction I, again indistinguishable from that of SFH, is shown in Fig. 3. The peaks are assigned to the heme (16 min), the $\beta$-globin (71 min) and the $\alpha$-globin (76 min) with the assignments being taken directly from Jeppson et al. [7]. Fig. 4 shows the chromatograms of the globin regions of the reversed-phase HPLC profiles of the individual components. None of the $\alpha$-globin peaks show appreciable change in either the intensity ($\alpha$/heme=0.3), the retention time or the width of the globin peak. The peaks assigned to the $\beta$-chains of fractions II, III and IV, however, are all both broadened and reduced in intensity. As well, an additional less mobile peak (83 min), presumably formed from $\beta$-globin, is observed in each of fractions II–IV. The chromatogram of fraction V contains a $\beta$ peak which is both more mobile (69 min) and significantly broadened. The less mobile $\beta$-globin peak (83 min), observed in fractions II–IV, is absent in the chromatogram of V. The reversed-phase HPLC profiles, therefore, indicate that the modification of Hb with o-ATP occurs predominantly on the $\beta$-chains.

## DISCUSSION

The results collectively show that fraction I is unreacted SFH. The major components of reaction products, fraction II, III and IV, contain far less phosphate than the amount required of 1:1 ATP–Hb adducts and exhibit no significant right shift in their oxygen dissociation curves. However, the differences in their anion-exchange and reversed-phase HPLC profiles relative to SFH indicate that they are indeed chemically modified. A reasonable rationale is that fractions II, III, and IV are comprised of Hb derivatives in which the ATP labels have lost their triphosphate groups. Simple hydrolytic loss may be ruled out because no ADP or

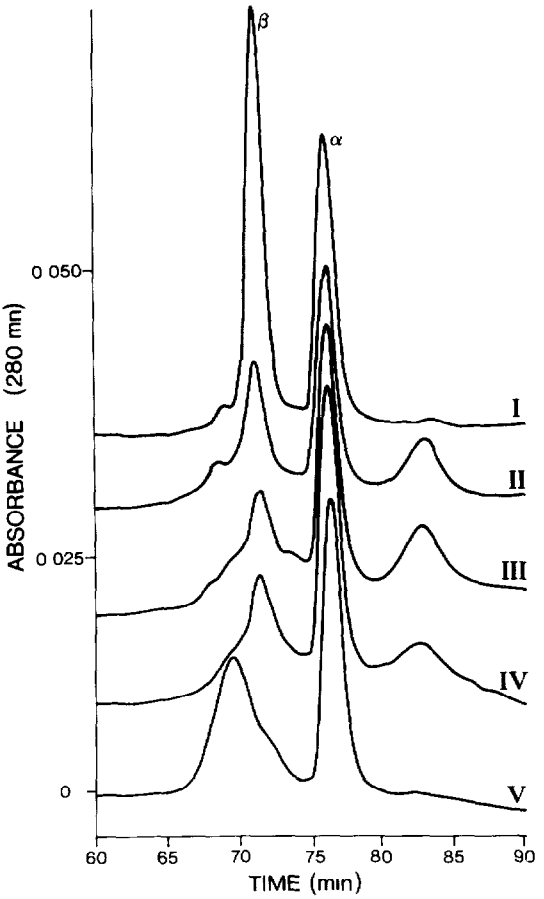


Fig. 4. The globin regions of the reversed-phase HPLC profiles of fraction I-V of the o-ATP-Hb reaction mixture obtained under the elution conditions described for Fig. 3.

AMP derivatives are observed. A reasonable hypothesis is then that the triphosphate moieties are lost via a $\beta$-elimination pathway. Since this reaction is well documented for o-ATP in aqueous solution [9], we see no reason to exclude o-ATP–Hb imines from a similar pathway under Scannon's reaction conditions [2]. Finally, the observed differences between fractions II, III and IV, in all likelihood, stem from differences in the labelling stoichiometry and locii.

A minor component of the mixture, fraction V, gives phosphate analyses that are indicative of an $(ATP)_2$ Hb adduct. The large right shift in its oxygen dissociation curve indicates that the ATP ligands impart a sizable allosteric effect and, therefore, probably occupy the hemoglobin diphosphoglycerate (DPG) binding site. The $\beta$-chain selectivity, as demonstrated in reversed-phase HPLC results, parallels the results of Benesch et al. [10] who found that $(PLP)_2$Hb, which also shows a right shift in its oxygen dissociation curve, is labelled exclusively on the 1-valine of the $\beta$-chains. Since these are the most reactive amino groups accessible in the DPG binding site [10], it seems reasonable to postulate

that the ATP labels in fraction V are probably bound to 1-valine residues of the $\beta$-chains.

Comparison of fraction V with ATP–Hb purified by the method Hsia et al. [3] is revealing. Briefly, Hsia et al. optimized Greenburg and Maffuid's [1] preparative procedures which, relative to Scannon's [2], differ mainly in that the o-ATP–Hb imines initially formed are reduced with sodium borohydride. Hsia et al. then used an ATP–agarose affinity gel to remove unreacted SFH from the reaction mixtures. The resulting ATP–Hb mixture, although complex, was found to have an average oxygen affinity comparable to that of fraction V ($P_{50}=35$ mmHg).

## CONCLUSION

In conclusion, the results indicate that the anomalously high oxygen affinity of ATP–Hb prepared by the method of Scannon [2] is observed simply because the bulk of the mixture is comprised of modified hemoglobins in which the triphosphate group has been lost, presumably via a $\beta$-elimination pathway. Borohydride reduction of the imines formed from o-ATP and Hb, as employed by Greenburg and Maffuid [1] and by Hsia et al. [3], evidently inhibits this process. It follows then that the potential of ATP–Hb as a blood substitute should not be minimized as a result of Scannon's observations. The reduced oxygen affinity of ATP–Hb and its prolonged plasma half-life make it by itself, and its polymerized derivatives [i.e., poly(ATP)–Hb], potentially viable alternatives to poly(PLP)–Hb [11] for use as hemoglobin-based blood substitutes.

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