Different Reactivities of Free and Bound Amino Groups in Deoxy- and Liganded Haemoglobin

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(Received 18 February 1977)

The technique of competitive double-labelling [H. Kaplan, K. J. Stevenson & B. S. Hartley, (1971) *Biochem. J.* **124**, 289–299; L. P. Visentin & H. Kaplan (1975) *Biochemistry* **14**, 463–468] was used to determine the reactivity of some amino groups towards acetic anhydride in deoxy- and liganded haemoglobin. Only those amino groups known to form salt bridges in deoxy- but not in liganded haemoglobin [i.e. the α -amino group of valine-1 α and the ϵ -amino group of lysine-40 α and lysine-127 α [M. F. Perutz (1970) *Nature (London)* **228**, 726–739]] had different reactivities in the two structures.

X-ray studies have shown that the quaternary structure of deoxyhaemoglobin is constrained by salt bridges that are absent from the quaternary structure of liganded haemoglobin. The ϵ -amino group of lysine-40 α_1 is linked to the carboxyl group of histidine-146 β_2 , that of lysine-127 α_1 to the carboxyl group of arginine-141 α_2 , and the α -amino group of valine-1 α to a Cl[−] ion (Fermi, 1975; Arnone *et al.*, 1976). The increase in pK of these amino groups should lower their reactivity towards any group attacking specifically their unprotonated form.

Kaplan *et al.* (1971) and Visentin & Kaplan (1975) have devised a competitive double-labelling method with acetic anhydride to determine the reactivities of amino groups in proteins as a function of their pK values and their microenvironment. I have used this method to measure the difference in reactivity of some amino groups in deoxy- and liganded haemoglobin.

Experimental

Human oxyhaemoglobin was prepared as described by Kilmartin & Rossi-Bernardi (1971) and passed through a column (5 cm × 20 cm) of Sephadex G-25 (fine grade) equilibrated with 0.4 M-sodium phosphate buffer, pH 7.01. The haemoglobin solution was diluted to a concentration of 1% with the same buffer and deoxygenated by repeated evacuation and flushing with N₂. A portion (1.61 ml) of this solution (containing 16.1 mg of haemoglobin) was transferred to a water-jacketed N₂-flushed cell maintained at 25°C and allowed 30 min, with moderate stirring, to achieve temperature equilibrium and complete deoxygena-

tion. Acetonitrile (10 μ l) containing 1.6 μ mol of [³H]acetic anhydride (sp. radioactivity 3.0 Ci/mmol) was added, and vigorous stirring was maintained throughout the 10 min during which the reaction was allowed to take place. No change in pH could be detected. The pH was lowered to 2.0 with 6 M-HCl, and 1.5 g of guanidinium chloride was added to a concentration of 6 M. After 15 min the pH of the solution was adjusted to 7.0 with 10 M-NaOH and the haemoglobin was completely acetylated by adding seven 12.5 μ l portions of [1-¹⁴C]acetic anhydride (1 mCi diluted to 300 μ l with undiluted acetic anhydride) at 5 min intervals. The solution was maintained at pH 7.0 ± 0.1 by manual addition of 10 M-NaOH from an Agla micrometer syringe. After complete reaction, 100 μ l of 5 M-NH₂OH, pH 6.0, was added, the pH adjusted to 6.0 with 6 M-HCl, and the reaction mixture was left for 1 h for complete hydroxylaminolysis of the O-acetyltyrosine residues (Smyth, 1967). The solution was dialysed against 0.05 M-ammonium bicarbonate and freeze-dried. Exactly the same procedure was used for carboxyhaemoglobin. The two freeze-dried samples were dissolved in 1 ml of 0.1 M-Tris/6 M-guanidinium chloride, pH 8.6, and 22 mg of dithiothreitol was added. After incubation for 1 h at room temperature (20°C) the samples were passed through a column of Sephadex G-25 (fine grade) equilibrated with the same buffer. Neutralized iodoacetic acid solution (90 μ l; 30 mg/ml) was added for complete carboxymethylation of the cysteine side chains. The reaction was allowed to proceed for 15 min and then stopped by adding 65 mg of dithiothreitol. After dialysis against 0.05 M-ammonium bicarbonate, the two samples were freeze-dried. Each sample was dissolved in 1% ammonium carbonate (making the solution 1% in protein) and digested with trypsin and chymotrypsin (1 mg of

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each enzyme per 50mg of protein) for 3h at 37°C. After digestion the pH of the two samples was adjusted to 3.5 with 2M-HCl, the precipitated haem was centrifuged, and the supernatants were freeze-dried. In this step 10% of the ^{14}C radioactivity was lost with the precipitated haem. The labelled peptides were purified by a combination of ionophoresis at pH 6.5, 3.5 and 2.1, and chromatography in butanol/acetic acid/water/pyridine (15:3:12:10, by vol.). On going from one purification procedure to the next, the peptides were located by radioautography on Kodinex Autoprocess AP5 film. After the final purification, the labelled peptides were eluted from the paper with 0.02M-NH₃. The radioactivity of a portion of the eluate was determined with a Nuclear-Chicago Unilux II liquid-scintillation counter with toluene/2-methoxyethanol (3:1, v/v) containing 2,5-diphenyloxazole (5g/l) and 1,4-bis-(5-phenyloxazol-2-yl)-benzene (0.25g/l). The remainder was hydrolysed with 6M-HCl in sealed evacuated tubes at 110°C for 24h, and the amino acid composition was analysed.

Results and Discussion

Table 1 lists the labelled lysines residues that were identified, the peptides in which they were present, and the $^3\text{H}/^{14}\text{C}$ ratios for any peptide, in the deoxy and liganded forms of haemoglobin. These ratios are a measure of the absolute reactivities of the amino groups (Visentin & Kaplan, 1975). The smaller the ratio, the lower the reactivity. The $^3\text{H}/^{14}\text{C}$ ratio of a peptide in deoxyhaemoglobin divided by that in liganded haemoglobin should be unity if the amino group(s) of this peptide do not change their pK values on binding of ligand. A ratio of less than 1 means that the pK of the amino group in deoxyhaemoglobin is higher than in liganded haemoglobin, as it would be

by formation of a salt bridge. These ratios are listed in Table 1. Only three are outside the average value of 1.00 ± 0.05 : they are the peptides containing valine-1 α , lysine-40 α and lysine-127 α , i.e. the ones described by Perutz (1970) as forming salt bridges in deoxy- but not in oxy-haemoglobin. The ratio for the peptide containing valine-1 α is higher (0.91) than those for lysine-40 α (0.73) and lysine-127 α (0.86), probably for the following reasons. First, the peptide α 1-9 also contains lysine-7 α ; if the ratio for lysine-7 α is unity, then the ratio for valine-1 α would be $2 \times 0.91 - 1.0 = 0.82$. Secondly, valine-1 α forms a salt bridge, not with the α -carboxyl group of arginine-141 of the opposite α -chain as suggested by Perutz (1970), but with a diffusible anion which in my experiments would have been phosphate (Arnone *et al.*, 1976). J. V. Kilmartin, A. Arnone & J. Fogg (unpublished work) found this anion site to be occupied for only part of the time, showing that its salt bridge with valine-1 α is weaker than the salt bridges made by the two lysine residues; hence the difference between the pK of valine-1 α in deoxy- and oxy-haemoglobin may be smaller. Lysine-127 α , shows abnormally low ratios of $^3\text{H}/^{14}\text{C}$ in liganded and unliganded haemoglobin, probably on account of its interaction with aspartic acid-6 α_1 in both quaternary structures. No other lysine residues show evidence of strong salt-bridging in the liganded form.

I attempted to use the same method for determining the lysine residues that form salt bridges between neighbouring molecules in fibres of deoxyhaemoglobin S, but was unable to obtain reproducible results, probably owing to technical difficulties.

I thank Dr. J. V. Kilmartin for suggesting and advising in this work, Dr. M. F. Perutz and Mrs. J. M. Baldwin for helpful discussions, and Mrs. J. Fogg for technical assistance.

Table 1. Summary of competitive-labelling data

Amino group	Peptide	$^3\text{H}/^{14}\text{C}$ ratio		(a)/(b)
		(a)	(b)	
		Deoxy-haemoglobin	Liganded haemoglobin	
Val-1 α , Lys-7 α	α 1-9	5.22	5.73	0.91
Lys-16 α	α 15-20	5.75	5.92	0.97
Lys-40 α	α 35-42	5.71	7.80	0.73
Lys-99 α	α 99-101	6.45	6.25	1.03
Lys-127 α	α 123-128	3.91	4.56	0.86
Lys-139 α	α 137-140	6.43	6.52	0.99
Val-1 β , Lys-8 β	β 1-14	6.00	6.10	0.98
Lys-17 β	β 16-30	7.17	7.56	0.95
Lys-59 β , Lys-61 β	β 56-63	5.74	5.78	0.99
Lys-65 β , Lys-66 β	β 64-68	5.63	5.53	1.02
Lys-120 β	β 119-122	6.52	6.29	1.04
Lys-132 β	β 131-139	5.71	5.57	1.02
Lys-144 β	β 142-145	6.61	6.58	1.00

References

- Arnone, A., O'Donnell, S. & Schuster, T. (1976) *Fed. Proc. Fed. Am. Soc. Biol.* **35**, 1604
- Fermi, G. (1975) *J. Mol. Biol.* **97**, 237–256
- Kaplan, H., Stevenson, K. J. & Hartley, B. S. (1971) *Biochem. J.* **124**, 289–299
- Kilmartin, J. V. & Rossi-Bernardi, L. (1971) *Biochem. J.* **124**, 31–45
- Perutz, M. F. (1970) *Nature (London)* **228**, 726–739
- Smyth, D. G. (1967) *J. Biol. Chem.* **242**, 1592–1598
- Visentin, L. P. & Kaplan, H. (1975) *Biochemistry* **14**, 463–468