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Instability of DTNB-treated Globin or Haemoglobin

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Abstract

Human haemoglobin or globin in its native form reacts with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) with uptake of two 3-carboxylato-4-nitrothiophenol groups, one for each of the reactive thiols at the $\beta 93$ positions.

Attempts to isolate the DTNB-treated globin by the acetone–HCl method, which unfolds the protein chains, result in disulphide interchange and oxidation of almost all the uncoupled 'masked' thiol groups. This modification is in marked contrast to the stability of haemoglobin or globin treated with reagents such as iodoacetic acid or *N*-ethylmaleimide that do not form disulphide bonds in blocking the thiol groups.

The derivatized globin chains have been separated by urea–thiol buffer chromatography on carboxymethylcellulose columns. Amino acid analysis and peptide mapping established the presence and location of disulphide bonds, whilst gel filtration in urea buffers and sodium dodecyl sulphate acrylamide gel electrophoresis defined the size of the products.

Introduction

Proteins were shown by Ellman (1959) to react rapidly with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and methods have been developed for using the reaction to estimate free thiol groups in native and denatured proteins (Habeeb 1972). In native proteins reaction is thought to be confined to thiol groups accessible on the surface, leaving other thiol groups unreacted due to their 'masking' in the hydrophobic interior. This reaction leads to formation of the thiol anion, 3-carboxylato-4-nitrothiophenylate (CNT), and a modified protein containing disulphide-linked CNT groups, often abolishing wholly or in part the activity of the molecule. The activity can usually be restored by incubation with a more powerful thiol such as dithiothreitol or mercaptoethanol.

In a previous paper (Nash and Thompson 1974) the presence of 2–4 reactive thiol groups in haemoglobin from the shark *Heterodontus portusjacksoni* was reported. Unless these groups are blocked aggregation occurs by disulphide bond formation. Reagents such as iodoacetic acid or *N*-ethylmaleimide that form irreversible covalent linkages resulted in much simpler electrophoretic patterns for the globin chains than did compounds like DTNB which form disulphide bonds.

In further study of this instability of DTNB-treated haemoglobin attention was given to the more readily available human haemoglobin, which has both reactive and unreactive thiol groups and gives similar complex electrophoretic patterns after DTNB treatment and removal of the haem group. Allison and Cecil (1958) showed

that only two of the six thiol groups of native human haemoglobin reacted with mercurial compounds. In the reactions with *N*-ethylmaleimide, iodoacetate or iodoacetamide (Benesch and Benesch 1962; Guidotti and Konigsberg 1964; Guidotti 1967) the reactive thiol groups were shown to be the β93 cysteine residues, the other thiol groups at β112 and α104 being inaccessible to the reagents. In support of this the structure of haemoglobin (Perutz *et al.* 1968) shows the involvement of the residues at β112 and α104 in subunit contacts that would bury them deep in the structure and make them inaccessible to thiol reagents. Birchmeier *et al.* (1972) have confirmed the greater reactivity of the β93 thiol groups in reaction with iodoacetamide and *p*-chloromercuribenzoate, there being only one reactive thiol group per dimer. In contrast to the normal $\alpha_2\beta_2$ tetramer, in the pathological human haemoglobin β_4 tetramer both thiol groups at β93 and β112 are reactive (Neer 1970).

There are reports in the literature that protein thiol groups estimated by DTNB may be qualitatively different from those reacting initially (Holbrook and Jeckel 1967; Boross 1969; Connellan and Folk 1969; Wasserman and Major 1969; Okabe *et al.* 1970; Telegdi and Straub 1973), suggesting instability of CNT groups. The advantage of a reversible blocking group for thiol groups in proteins may be lost if secondary reactions occur.

Materials and Methods

Separation of α and β Components of Normal and DTNB-reacted Haemoglobin

Separations were effected on columns (10 by 2 cm) of carboxymethylcellulose according to the method of Clegg *et al.* (1966). In the fractionation of the DTNB-reacted globin, mercaptoethanol was rigorously excluded from the buffers. Elution gradients of 10–50 mM Na^+ in phosphate buffer (pH 6·7) were routinely used.

Electrophoresis

Cellulose acetate electrophoresis was on Cellogel in 0·1 M tris–HCl–0·001 M EDTA buffer, pH 8·6, at 200 V for 30–60 min. To separate the α- and β-globin chains the strip was pre-saturated with buffer made 8 M in urea as previously described (Nash and Thompson 1974).

Disc electrophoresis on polyacrylamide gels in sodium dodecyl sulphate solutions was carried out according to Weber *et al.* (1972).

Amino Acid Analysis and Peptide Mapping

Quantitative analyses were carried out using a Beckman 120C amino acid analyser on hydrolysates prepared in sealed evacuated tubes at 110°C for 24 h. Qualitative analyses were carried out by paper ionophoresis at pH 1·8 (Air and Thompson 1969).

Tryptic digestion and mapping of the peptides were by standard methods as described by Thompson *et al.* (1969).

For analysis of disulphide bonds the protein was reduced at pH 8·0 with 40 mM dithiothreitol in 6 M guanidine hydrochloride and carboxymethylated at pH 8·5 with an equivalent amount of iodoacetic acid followed by recovery and analysis of the protein for *S*-carboxymethylcysteine (Konigsberg 1972). Control experiments used guanidine hydrochloride but without added dithiothreitol.

Thin-layer Gel Filtration of Proteins

Gel filtration was carried out on thin layers according to the method of Morris (1964) with some modifications (Radola 1968). Sephadex G100 superfine was first swollen in pH 6·7 phosphate buffer and urea added subsequently to 8 M concentration. Myoglobin from kangaroo was used as reference protein.

Results

After reaction of haemoglobin with DTNB and isolation of the globin by acetone–HCl precipitation, the numbers of -SH and CNT groups in the globin were determined and are shown in Table 1 and compared with the results obtained after reaction of *N*-ethylmaleimide with haemoglobin. In the case of *N*-ethylmaleimide-treated protein there are approximaetly four unreacted thiol groups, and further treatment of the product with DTNB did not lead to loss of thiol groups. With the DTNB-treated haemoglobin, however, a globin product was formed with no thiol groups in stoichiometric amounts and a maximum of two CNT groups per mole.

Table 1. Content of -SH and CNT groups in globin isolated after treatment of haemoglobin with DTNB or *N*-ethylmaleimide

NEM, *N*-ethylmaleimide. HB, haemoglobin

Mol DTNB/ mol HB	Mol SH/mol globin tetramer	Mol CNT/mol globin tetramer	Mol NEM/ mol HB	Mol SH/mol globin tetramer	Mol CNT/mol globin tetramer
0	6·1	0	0	6·2	—
1	0·2	1·0	2	4·5	0
2	0·3	2·0	6	4·2	0
6	0·4	2·0			
8	0·6	—			

The DTNB-treated haemoglobin was stable in solution and could be dialysed free of excess reagent and CNT^- ion. This product moved as a single discrete band on ionophoresis, slightly more negatively charged at pH 8·6 than untreated haemoglobin. If treated with mercaptoethanol in dilute solution CNT^- ions were liberated (2 mol/tetramer) with regeneration of normal haemoglobin. These results, in conjunction with the lack of significant changes in the positions of the absorption maxima in the spectra of the haemoglobin and DTNB-treated haemoglobin, suggested that no marked changes in native conformation occurred during the initial reaction of haemoglobin with DTNB.

With native globin which has recovered its original conformation after acetone–HCl precipitation (Rossi Fanelli *et al.* 1958) the increase in absorption at 412 nm could be followed. Two thiol groups per tetramer were titrated with DTNB. When this CNT-globin was precipitated under the mild conditions of Rossi Fanelli *et al.* (1958) the bulk of the CNT-globin, insoluble at pH 8, contained 1·5 molar proportions of CNT groups whilst the smaller amount soluble at pH 8 contained 4 molar proportions of CNT groups. This indicates that a redistribution of CNT groups has occurred.

Typical fractionations of normal human globin and globin prepared from DTNB-treated haemoglobin (2 mol DTNB/tetramer) on carboxymethylcellulose columns at pH 6·7 in 8 M urea are shown in Fig. 1. On addition of mercaptoethanol, fractions carrying the CNT group yielded a yellow colour measurable at 412 nm. Solutions of the globin from DTNB-treated haemoglobin in the 8 M urea buffer released some CNT^- ions which appeared as a yellow band passing straight through the column and emerged in the same fraction as the small haem pigment peak in the case of normal haemoglobin. This yellow front intensified further on addition of mercapto-ethanol and contained traces of protein which did not bind to the carboxymethyl-cellulose under the conditions used. Apart from this the globin from the DTNB-

treated haemoglobin yielded four fractions corresponding to peaks 1, 2, 3 and 4 (Fig. 1), two emerging in the positions corresponding to the β- and α-chains of normal globin and two emerging earlier than the main β-chain. The two early peak fractions 1 and 2 and the peak fraction 4 at the α-chain position carried CNT groups as shown by treatment with mercaptoethanol. Fraction 2 was much smaller than fraction 1 and contained fewer CNT groups. A final peak 5 was eluted from the column with more concentrated buffer and contained no CNT groups.

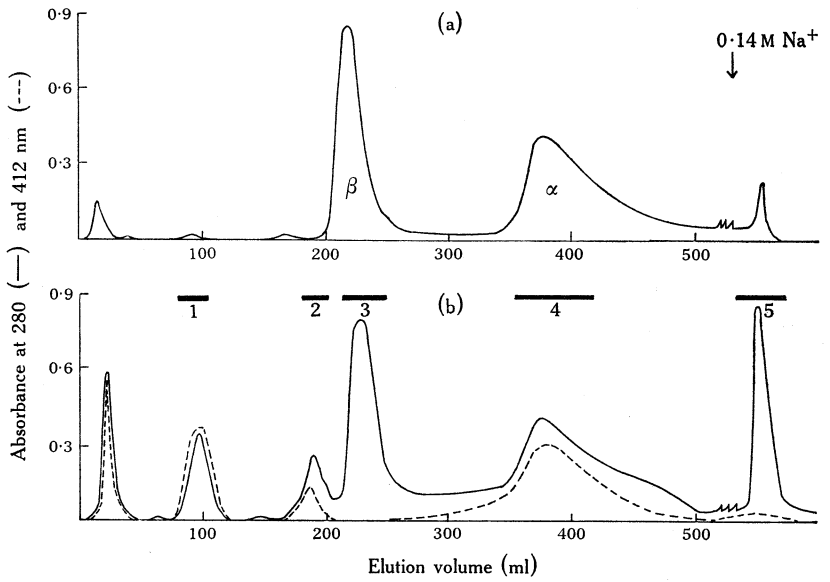

Fig. 1. Separation of the subunits of (*a*) normal human globin and (*b*) globin from DTNB-reacted human haemoglobin by carboxymethylcellulose chromatography. A 10 by 2 cm column of carboxymethylcellulose was equilibrated with 8 M urea–10 mM Na^+ (phosphate)–1 mM EDTA buffer, pH 6·7. Approximately 100 mg of the globin dissolved in buffer was applied and washed in with a few millilitres of the same buffer. A linear Na^+ gradient was then applied at an elution rate of 60 ml/h, using 8 M urea–50 mM Na^+ (phosphate)–1 mM EDTA (pH 6·7) as limit buffer. Mercaptoethanol at a concentration of 50 mM was included in all buffers used for separation of normal globin; it was rigorously excluded from the medium in the case of the derivatized globin. The broken line in (*b*) represents absorption at 412 nm after treatment of the effluent with mercaptoethanol.

Tryptic digestion and peptide mapping of the protein corresponding to each peak showed that the two fractions emerging prior to the peak in the normal β-chain position were β-chain derivatives; no α-chain tryptic peptides were found in either of these β-chain derivatives in the several preparations that were made.

As seen in Fig. 1 the elution of the α-chains of normal globin occurred well after the emergence of the β-fractions, the two fractions separating completely with no trace of the tryptic peptides of the β-globin in the α-fraction or vice versa. In contrast, the fraction 4 of DTNB-treated globin which eluted after β-globin, in the approximate position that normal α-chain would emerge, was found to give a peptide map corresponding to an α–β composition (designated CNT-αβ fraction). Elution and analysis of the peptides formed by tryptic digestion and peptide mapping revealed

all the expected peptides, except the core peptides β_{12} and α_{12-13}, and the β_{10+11} segment of the molecule. The latter peptides β_{10}, β_{10+11} and β_{11} are normally present in β-chain peptide maps (Hill *et al.* 1962). Amino acid analysis of fraction 4 before and after reduction with dithiothreitol confirmed the presence of disulphide bonds in this fraction and of α-globin and β-globin in approximately equal proportions, rather than a small degree of contamination of one chain with another. When a peptide map of the CNT-$\alpha\beta$ fraction 4 was sprayed with mercaptoethanol in buffer of pH 8 (Glaser *et al.* 1970) no yellow spots corresponding to the positions of any of the peptides could be detected. Since the presence of CNT groups in this fraction could be demonstrated it can be concluded that the CNT chromophore was attached to an insoluble 'core' peptide fraction, probably β_{10} or β_{10+11}.

Peptide maps of the most strongly adsorbed fraction 5, eluted as a sharp peak with 0·14 M Na^+–urea buffer, showed that it consisted of α-chain peptides with only traces of β-chain contamination.

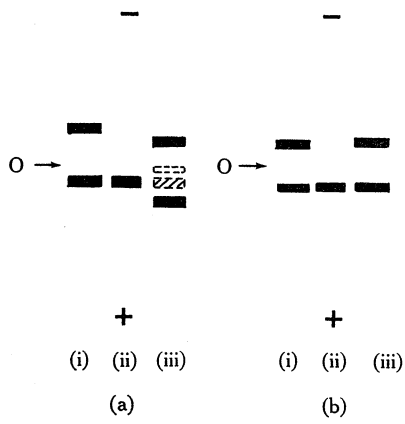

Fig. 2. Electrophoretic patterns of (*a*) isolated and (*b*) mercaptoethanol-treated normal human globin (i) and fractions 3 (ii) and 4 (iii) from the carboxymethylcellulose chromatography of globin prepared from DTNB-reacted human haemoglobin. Electrophoresis was carried out for 60 min as described in Methods. O denotes the origin. The dashed and cross-hatched zones were minor products.

Cellogel electrophoresis in 8 M urea buffer of fractions 3 and 4 in parallel with normal globin (Fig. 2) showed that fraction 3 contained only one component corresponding to the β-chain position whilst fraction 4, judging from the intensity of staining, was slowly resolved into two components with mobilities slightly different from those of α- and β-chains. The β-chain band from fraction 4 was more electronegative (anodic) than normal β-globin, and the α-chain band slightly less electropositive (cathodic) than normal α-globin. Fraction 4, after treatment with mercaptoethanol, gave an electrophoretic pattern (Fig. 2) identical with that of normal $\alpha_2\beta_2$ globin, suggesting that the presence of CNT groups was affecting the mobilities of both chains.

The absence of CNT groups in fraction 3 suggests that the normal thiol groups in the β-chain have been oxidized to the disulphide form. Quantitative amino acid analysis of the untreated and of reduced and carboxymethylated fractions 3 and 4 yielded the values shown in Table 2 for cystine and *S*-carboxymethylcysteine respectively.

Mobilities of the various fractions on Sephadex G100 thin layer plates developed with urea buffers revealed that fraction 1 was a mixture of species with molecular weights of 17 000 and 34 000, fraction 2 and fraction 4 were approximately 34 000,

fraction 3 was about 17 000, and fraction 5 was mainly 34 000 with a small amount of 65 000 and higher-molecular-weight species. The results on sodium dodecyl sulphate polyacrylamide gels with and without reduction by mercaptoethanol confirmed these findings. The sodium dodecyl sulphate gels showed that both fraction 4 (mainly α-S-S-β-S-CNT) which gave an α plus β peptide map, and fraction 5 (α-S-S-α) which gave an α peptide map, had molecular weights of 34 000. Fraction 3, the main fraction containing β-chain, had a molecular weight of 17 000 indicating that the disulphide bond linked the two β-thiol groups intramolecularly.

Table 2. *S*-Carboxymethylcysteine and cystine content of fractions from carboxymethylcellulose chromatography of globin prepared from DTNB-treated haemoglobin

Samples of fractions 3 and 4 were analysed after carboxymethylation and after reduction with dithiothreitol followed by carboxymethylation. Values are expressed as residues per expected content of phenylalanine

Amino acid	Isolated fraction 3	Reduced, carboxy-methylated fraction 3	Theoretical content of β-chain	Isolated fraction 4	Reduced, carboxy-methylated fraction 4	Theoretical content of α-S-S-β
Phenylalanine	8·0	8·0	8	15	15	15
½ Cystine	1·3	0	0	+ve	0	2
SCM-cysteine	0	1·7	2	0	2·6	3

Discussion

When native globin is prepared and treated with DTNB at pH 8 there is a liberation of a maximum of 2 mol CNT^-, indicative of two reactive thiols. This results in formation of a compound that is stable in solution with a somewhat increased negative charge due to the carboxyl groups of the disulphide-linked CNT groups. When this CNT-globin was precipitated from solution under the mild conditions of Rossi Fanelli *et al.* (1958) there was a redistribution of CNT groups between the CNT globin fractions that were soluble and insoluble at pH 8. This indicates that interchange reactions are occurring during acetone–HCl precipitation, a process that involves denaturation.

When haemoglobin is treated with DTNB there is a similar uptake of two CNT groups by the β93 thiol groups. When this DTNB-treated haemoglobin is precipitated as globin in acetone-HCl by the method of Anson and Mirsky (1930) the CNT groups are not stable. Disulphide interchange as well as oxidative reactions occur since not only are several fractions obtained containing CNT groups but almost complete loss of the expected four thiol groups occurs. Normally thiol groups are most stable at low pH values. The disulphide-linked CNT groups act catalytically in these changes and one mole proportion per mole haemoglobin tetramer is sufficient to lead to this loss of thiol groups.

$$\text{SH-protein-S}^- + \text{DTNB} \rightarrow \text{SH-protein-S-CNT} + \text{CNT}^-$$

$$\text{SH-protein-S-CNT} \rightarrow \overline{\text{S-Protein-S}} + \text{CNT}^- + \text{H}^+$$

$$2\text{CNT}^- + 2\text{H}^+ + \tfrac{1}{2}\text{O}_2 \rightarrow \text{DTNB} + \text{H}_2\text{O}$$

The initial reaction of the β93 thiol groups with DTNB appears to be essential to these oxidative changes. If *N*-ethylmaleimide is reacted with haemoglobin to couple with the β93 thiols then no further reaction occurs with DTNB and four thiol groups are present in the recovered *N*-ethylmaleimide-globin following this treatment with DTNB.

The conclusion that loss of conformation induces disulphide interchange reactions when the less stable disulphide-linked blocking groups like DTNB are used is probably the explanation for the release of CNT^- ions when the DTNB-derivatized globins are dissolved in 8 M urea buffer at pH 6·7, in spite of the absence of any added reducing agent. There are some thiol groups, in non-stoichiometric amounts, in the precipitated globin that can undergo exchange reactions.

Although the iron-containing haem group makes haemoglobin atypical, the results with native globin and reports in the literature by Okabe *et al.* (1970) in the case of creatine kinase together with the postulated intramolecular disulphide bond formation leading to release of CNT^- ions for some enzymes (Wassarman and Major 1969; Connellan and Folk 1969) suggests that the results with DTNB-globins may be similar in other proteins. Certainly if denaturation conditions prevail as in the experiments reported in this paper, catalysed disulphide interchange reactions seem likely to result in the final location of the CNT groups being different from the initial thiol groups reacted, and all possible disulphide-linked chains can be expected.

The reactions envisaged are consistent with the work of Weitzman (1975) who showed that disulphide bonds once formed in proteins are not cleaved by the CNT^- ion.

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References

Air, G. M., and Thompson, E. O. P. (1969). *Aust. J. Biol. Sci.* **22**, 1437–54.
Allison, A. C., and Cecil, R. (1958). *Biochem. J.* **69**, 27–34.
Anson, M. L., and Mirsky, A. E. (1930). *J. Gen. Physiol.* **13**, 469–76.
Benesch, R. E., and Benesch, R. (1962). *Biochemistry* **1**, 735–8.
Birchmeier, W., Glatthaar, B. E., Winterhalter, K. H., and Bradshaw, R. A. (1972). *Eur. J. Biochem.* **28**, 533–7.
Boross, L. (1969). *Acta Biochim. Biophys. Acad. Sci. Hung.* **4**, 57–69.
Clegg, J. B., Naughton, M. A., and Weatherall, D. J. (1966). *J. Mol. Biol.* **19**, 91–108.
Connellan, J. M., and Folk, J. E. (1969). *J. Biol. Chem.* **244**, 3173–81.
Ellman, G. L. (1959). *Arch. Biochem. Biophys.* **82**, 70–7.
Glaser, C. B., Maeda, H., and Meienhofer, J. (1974). *Int. J. Peptide Protein Res.* **6**, 223–30.
Guidotti, G. (1967). *J. Biol. Chem.* **242**, 3673–84.
Guidotti, G., and Konigsberg, W. (1964). *J. Biol. Chem.* **239**, 1474–84.
Habeeb, A. F. S. A. (1972). In 'Methods in Enzymology'. (Eds C. H. W. Hirs and S. N. Timasheff.) Vol. XXV Part B. pp. 457–64. (Academic Press: New York.)
Hill, R. L., Swenson, R. T., and Schwartz, H. C. (1962). *Blood* **19**, 573–86.
Holbrook, J. J., and Jeckel, R. (1967). *Arch. Biochem. Biophys.* **122**, 519–21.
Konigsberg, W. (1972). In 'Methods in Enzymology'. (Eds C. H. W. Hirs and S. N. Timasheff.) Vol. XXV Part B. pp. 185–8. (Academic Press: New York.)
Morris, C. J. O. R. (1964). *J. Chromatogr.* **16**, 167–75.

Nash, A. R., and Thompson, E. O. P. (1974). *Aust. J. Biol. Sci.* **27**, 607–15.
Neer, E. J. (1970). *J. Biol. Chem.* **245**, 564–9.
Okabe, K., Jacobs, H. K., and Kuby, S. A. (1970). *J. Biol. Chem.* **245**, 6498–510.
Perutz, M. F., Muirhead, H., Cox, J. M., and Goaman, L. C. G. (1968). *Nature (London)* **219**, 131–9.
Radola, B. J. (1968). *J. Chromatogr.* **38**, 61–77.
Rossi Fanelli, A., Antonini, E., and Caputo, A. (1958). *Biochim. Biophys. Acta* **30**, 608–15.
Telegdi, M., and Straub, F. B. (1973). *Biochim. Biophys. Acta* **321**, 210–19.
Thompson, E. O. P., Hosken, R., and Air, G. M. (1969). *Aust. J. Biol. Sci.* **22**, 449–62.
Wassarman, P. M., and Major, J. P. (1969). *Biochemistry* **8**, 1076–82.
Weber, K., Pringle, J. R., and Osborne, M. (1972). In 'Methods in Enzymology'. (Eds C. H. W. Hirs and S. N. Timasheff.) Vol. XXV Part C. pp. 3–27. (Academic Press: New York.)
Weitzman, P. D. J. (1975). *Biochem. J.* **149**, 281–3.

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