^{1}H NMR of albumin in human blood plasma: drug binding and redox reactions at Cys^{34}

John Christodoulou, Peter J. Sadler*, Alan Tucker

Department of Chemistry, Birkbeck College, University of London, Gordon House, 29 Gordon Square, London WC1H 0PP, UK

Received 10 August 1995; revised version received 4 October 1995

Abstract **^{1}H NMR methods are described which allow direct studies of the Cys^{34} binding site of albumin in intact human blood plasma in vitro. Antiarthritic gold drugs and the alcohol-aversive drug disulfiram induce a structural transition detectable via Hε1 and Hδ2 resonances of His^3 of albumin, and reactions of cystine, glutathione and captopril in plasma have also been investigated. Contrary to most assumptions, little of the albumin in normal plasma appears to be blocked at Cys^{34} as a cystine disulfide.**

Key words: Blood plasma; Albumin; ^{1}H NMR; Gold drug; Disulfide; Thiol

1. Introduction

Albumin (66.5 kDa) is a non-glycosylated, single-chain protein of 585 amino acids with 17 disulphide bridges and one free thiol at Cys^{34}; with a concentration in blood of ca. 0.63 mM. It is the major transport protein in blood plasma for drugs, as well as for fatty acids, hormones, metal ions and xenobiotics [1,2]. However, the structural and dynamic interactions of these substances with albumin are, in general, poorly understood and this is hampering the rational design of many drugs and diagnostic agents [3].

Strong binding sites on albumin include: I and II for small organic molecules, III and IV for long-chain fatty acids, V Cys^{34}, and VI the square-planar N-terminal Cu^{2+} and Ni^{2+} site [4,5]. X-Ray crystallographic studies have recently elucidated the selectivity of sites I (e.g. for warfarin) and II (e.g. for diazapam, digitoxin) in subdomains IIA and IIIA, respectively, and detected movements of helices when fatty acids bind in sites III and IV. Albumin has not been crystallized with site VI occupied, but this metal site has been characterized in solution [6] and in model peptides [7]. In contrast, binding and redox processes at site V, Cys^{34}, are much less well characterized despite intense interest over many years (e.g. [8]). In the crystal structure of albumin, Cys^{34} is protected in a crevice where it interacts with other side-chains, probably His^{39} and Glu^{82} [5]. This may allow stabilization of the deprotonated thiol and account for its low pK_a of between 5 and 7 [9,10].

We report here that redox chemistry (including disulfide interchange reactions) and drug binding at site V (Cys^{34}) can be detected directly in human blood plasma in vitro by ^{1}H NMR spectroscopy. It is proposed that binding to this site may be accompanied by a 'flip-out' of Cys^{34}. Although it is often assumed that 30–40% of albumin molecules in circulation have Cys^{34} blocked as a disulfide [11], our experiments suggest that little of the albumin in fresh normal plasma is present in this form. The NMR approach reported here should find widespread application in studies of the redox balance in blood plasma and the design of novel drugs and other agents.

2. Materials and methods

2.1. Materials

L-Cystine, disulfiram (*N*,*N*-diethyldithiocarbamate disulfide), glutathione (γ-L-Glu-L-Cys-Gly) disulfide and captopril (D-3-mercapto-2-methylpropanoyl-L-proline) were purchased from Sigma. Auranofin (triethylphosphine-(2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyrano-sato-S)gold(I)) and aurothiomalate (disodium gold(I) thiomalate) were the gifts of SmithKline Beecham and Rhône Poulenc, respectively. These were added to plasma as aliquots of concentrated aqueous solutions, except for auranofin which was added in methanol.

2.2. Sample preparations

Blood from a healthy male volunteer was collected into lithium heparin vials and centrifuged at 277 K to remove cells, and then treated in one of two ways. For samples in 95% H_2O/5% D_2O, 50 μl of 0.2 M NaH_2PO_4 (in D_2O) was added to 0.95 ml of plasma, whereas for work with D_2O samples, plasma was freeze-dried immediately in 0.5 ml aliquots and reconstituted in half the original volume of NaH_2PO_4 (10 mM) in D_2O as required. High M_r fractions were obtained by gel permeation on PD10 (Pharmacia) columns prior to freeze drying. All experiments were carried out with as fresh plasma as possible which was normally used within 24 h of collection. The normal albumin concentration in plasma was assumed to be 0.63 mM [1].

2.3. pH measurements

Measurements of pH or pH* (pH meter reading in D_2O solutions) were made using a Corning 145 pH meter equipped with an Aldrich micro combination electrode calibrated with Aldrich pH buffers at pH 4, 7 and 9. Adjustments of pH were made with microlitre aliquots of 1.0 M NaOD or DCl. Careful control of pH is very important in this work since many His resonances are very sensitive to small pH changes in the physiological range.

2.4. NMR spectroscopy

600 MHz ^{1}H NMR spectra were recorded at 310 K on Varian VXR600 (Biomedical NMR Centre, Mill Hill) or Bruker AMX600 (ULIRS NMR service, Queen Mary and Westfield College) instruments, and 500 MHz spectra on a JEOL GSX500 spectrometer (ULIRS Biomedical NMR Centre, Birkbeck College), using 0.7 or 0.5 ml of sample in 5 mm tubes. Typical conditions: 40–50° pulses, spectral width 12 ppm, 8 K data points, acquisition time 0.5–0.7 s, relaxation delay 2–2.3 s, 512–1024 transients. For samples in D_2O, residual HOD was suppressed when necessary by presaturation. For samples in 95% H_2O/5% D_2O the WATERGATE sequence [20], which incorporates tailored excitation and pulsed-field-gradients, was used to suppress the intense solvent resonance. An optimal combination of exponential (1.0 Hz line-broadening) and unshifted sine-bell functions [13] was used for resolution enhancement and free induction decays were zero-filled once before Fourier transformation. Chemical shifts are referenced to sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (TSP) via formate (8.48 ppm; present in all samples).

*Corresponding author. Fax: (44) (171) 380 7464.
E-mail: ubca424@ccs.bbk.ac.uk

SSDI 0014-5793(95)01231-1

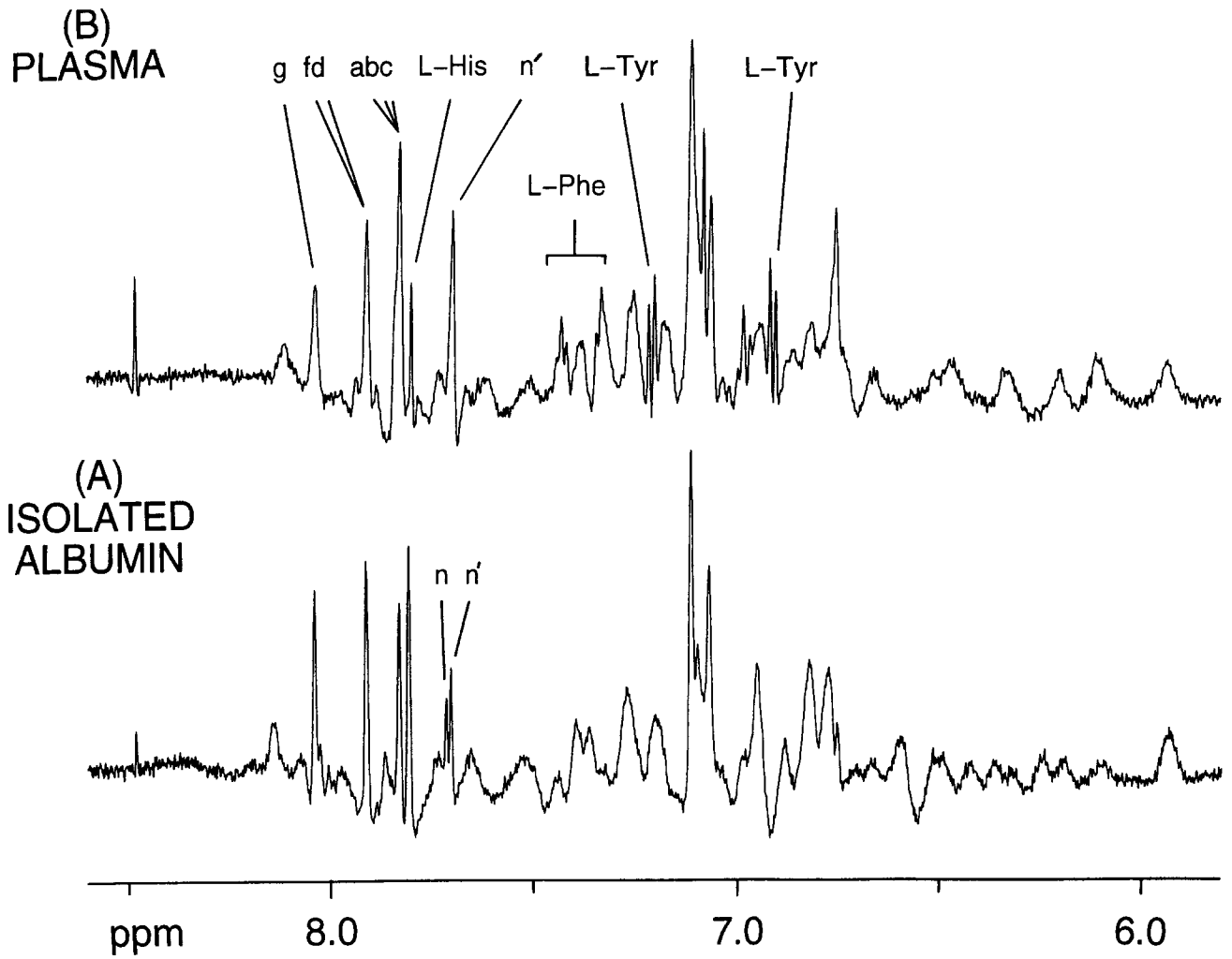


Fig. 1. 600 MHz ^{1}H NMR spectra of the aromatic region of (A) a commercial sample of clinically-used (isolated) human albumin (Armour Albuminar, dialyzed, freeze-dried, and reconstituted into deuteriated 10 mM phosphate buffer pH* 7.4) and (B) fresh human blood plasma (freeze-dried, reconstituted in the same buffer). Assignments: n, n′ Hε1 of His^3 of albumin with Cys^{34} in blocked and thiolate forms, respectively; a to k, other His Hε1 resonances of albumin; L-Phe, L-Tyr and L-His, free amino acids. Note the high n:n′ ratio in (A) compared to (B).

3. Results

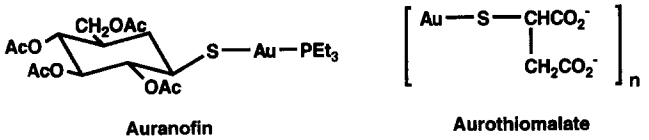
We have found that reactions at Cys^{34} of albumin in blood plasma can be detected via observation of ^{1}H NMR resonances assignable to His^3.

3.1. Assignment of His resonances of albumin in spectra of plasma

Many peaks in the aromatic region (6.5 to 9.5 ppm) of a typical (resolution-enhanced) ^{1}H NMR spectrum of human blood plasma are similar to those of isolated albumin, Fig. 1. The assignment of peaks to His residues of albumin was achieved by pH titrations. Previous studies on human albumin [13,14] and recombinant wild-type and mutant albumins (Christodoulou, J., Sadler, P.J. and Tucker, A. unpublished) have shown that His Hε1 resonances have characteristic pH titration profiles, in particular that for His^3. Similar titration profiles were obtained for 10 resonances in the aromatic region of spectra of blood plasma, Fig. 2A. Values of pK_a were obtained from curves fitted to the data, and Fig. 2B shows the excellent correlation between the pK_a values determined for ten His residues of albumin in plasma with those of isolated albumin. The assignment for His^3 Hε1 (pK_a 6.42) in the spectrum of plasma agrees with that based on Ni(II) binding at site VI [15], and is supported by the absence of this peak in spectra of albumins that lack His^3 (pig, dog [13]), and its perturbation in spectra of albumin with Asp^1 removed [16].

3.2. Drug binding to albumin in blood plasma

Initially we studied reactions of the antiarthritic gold drugs aurothiomalate (Myocrisin) and auranofin (Ridaura) with fresh plasma reconstituted in deuteriated buffer. Albumin is known to carry more than 70% of the circulating gold in patients treated with these drugs [17], and the major binding site for gold(I) on albumin is known to be Cys^{34} [18]. Addition of either auranofin, or aurothiomalate (data not shown), to blood plasma at pH* 7.4, appeared to give rise to a quantitative conversion of His^3 Hε1 peak n′ into peak n, Fig. 3.



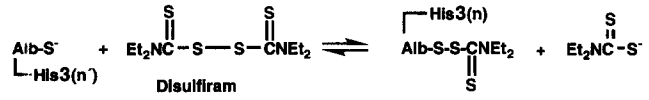
Scheme 1.

In contrast, addition of one mol equivalent of auranofin (with respect to albumin) to blood plasma at pH* 6.1 produced only a ca. 50% switch in n′ to n intensities. It is also notable that the spectrum of plasma alone shown in Fig. 3 shows no evidence of peak n (suggesting that the blocked form of albumin is not present, vide infra).

The same structural switch involving His^3 of albumin in blood plasma was also observed when disulfides were added. These can block Cys^{34} via interchange reactions:

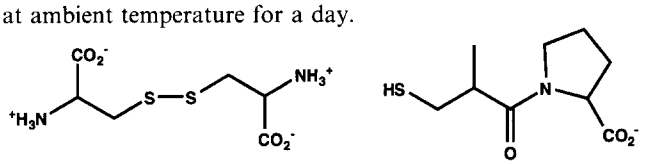
$$Alb\text{-}Cys^{34}\text{-}S^- + RSSR = Alb\text{-}Cys^{34}\text{-}S\text{-}S\text{-}R + RS^-$$

Addition of the drug disulfiram (Antabuse) to plasma caused a rapid and quantitative switch of Hε1 peak of His^3 from n′ to n consistent with formation of blocked albumin and release of diethyldithiocarbamate (reduced drug):



Scheme 2.

Similarly addition of the disulfide L-cystine (0.5 and 1 mol equiv with respect to albumin) to plasma also gave rise to the His^3 n′ to n peak switch (data not shown), consistent with formation of 'Cys-blocked' albumin (Alb-'Cys^{34}'-S-S-Cys), and free L-Cys. This was very clearly detectable from both the Hε1 and Hδ2 peaks of His^3. Interestingly addition of similar amounts of either glutathione disulfide or D-penicillamine disulfide, the primary metabolite of the drug D-penicillamine, did not cause the His^3 structural switch after incubation in plasma for several hours at 310 K. We also studied in detail reactions of L-cystine with the high M_r (>5 kDa) fraction of plasma since spectra are simpler, avoiding overlap of albumin peaks with those of low M_r molecules. Again peak n for His^3 Hε1 was absent in the initial spectrum and only peak n′ was seen, but peak n appeared after reaction with added L-cystine. The latter peak also appeared in the spectrum of the control sample left at ambient temperature for a day.



Scheme 3.

Reversal of disulfide blockage of albumin in plasma was also studied. Cys-blocked albumin was generated in blood plasma by reaction with L-cystine and the n′ → n switch was observed. Subsequent addition of the antihypertensive thiol drug captopril (0.5 mol equiv), an inhibitor of angiotensin I-converting enzyme, caused a rapid reversal (< minutes – within time taken to record spectrum) of the His^3 structural switch.

All of the above NMR experiments were carried out with fresh blood plasma which had been freeze-dried, reconstituted in D_2O and preconcentrated by a factor of two to allow acquisition of ^1H NMR spectra with signal-to-noise ratios good enough for resolution enhancement within a reasonable period of time. This procedure also simplifies data acquisition by suppression of the intense H_2O peak and spectral overlap in the aromatic region by removal of NH resonances [19]. However, we were also able to show that experiments can be carried out on intact plasma without these pretreatment steps using water suppression via a combination of tailored excitation and pulsed-magnetic-field gradients (WATERGATE [20]). On account of the low concentration of albumin, longer spectral accumulation times (ca. 1 h) are needed. Similar results are obtained, as shown by the disulfiram-induced n′ → n switch illustrated in Fig. 4. These data show that our deuteration procedures did not introduce artifacts.

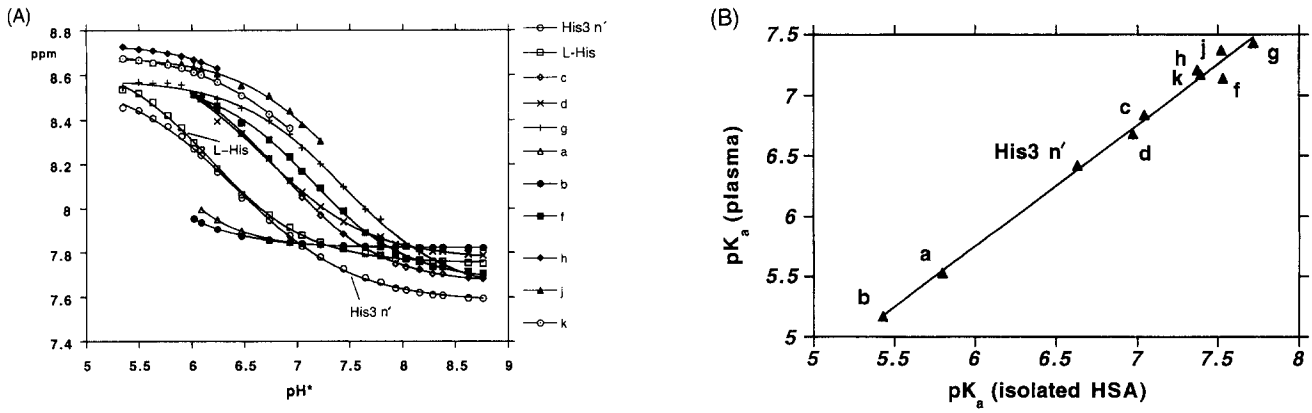


Fig. 2. Determination of pK_a values for His residues of albumin and L-His in blood plasma. (A) Variation of chemical shifts of His Hε1 resonances with pH*. The pK_a values, limiting chemical shifts (δ_A, δ_{HA}), and Hill coefficients (n) were extracted from the fitted curves using one of three functions: (i) $\delta_{obs} = (([H^+]^n \cdot \delta_{HA}) + (K_a^n \cdot \delta_A))/([H^+]^n + K_a^n)$ for complete data (ii) $\delta_{obs} = (([H^+]^n \cdot (\delta_A + 0.9)) + (K_a^n \cdot \delta_A))/([H^+]^n + K_a^n)$ for data incomplete at low pH*, and (iii) $\delta_{obs} = (([H^+]^n \cdot \delta_{HA}) + (K_a^n \cdot (\delta_{HA} - 0.9)))/([H^+]^n + K_a^n)$ for data incomplete at high pH*. (B) Plot of pK_a values of His residues for isolated human albumin, calculated from reported data [12] using the fitting procedure described above, versus those determined for plasma. The gradient is 1.00, correlation coefficient 0.996, and intercept of −0.28, the latter presumably arising from differences in ionic strength and temperature of the measurements. The pK_a for L-His in plasma was determined to be 6.18.

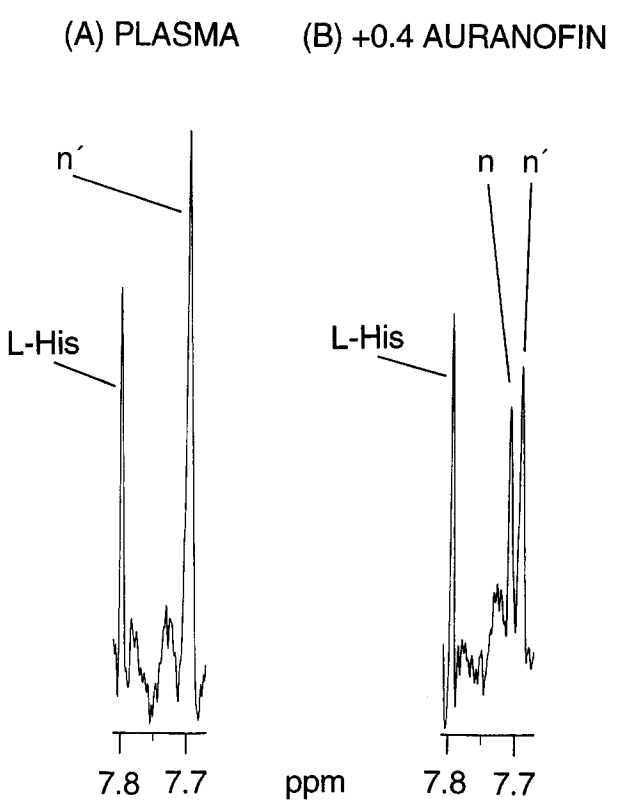


Fig. 3. 600 MHz ^{1}H NMR spectra of the His3 Hε1 region of human blood plasma pH* 7.4. (A) Normal plasma in D_2O. (B) Plus 0.4 mol equiv (with respect to albumin) of the oral antiarthritic drug auranofin. The n′ → n switch was complete with ca. 1 mol equiv of added auranofin (not shown). Auranofin reacts with Cys^{34} to form Alb-'Cys^{34}'-S-Au-PEt$_3$. The same n′ → n switch is seen when Cys^{34} is oxidized, e.g. formation of a disulfide with L-cystine. It can therefore be concluded that there is little blocked Cys^{34} present in fresh plasma.

4. Discussion

On the basis of the data presented above for both Au(I) binding to albumin in plasma and for disulfide interchange reactions, it is clear that little (<10%) of the albumin in normal fresh plasma is present in the structurally-switched (n) form which is characteristic of the blocking of Cys^{34}. This is clear from the spectrum of normal plasma shown in Fig. 3A which lacks peak n for His^3. In contrast, peak n is readily detected when Cys-blocked albumin is artificially generated in plasma by addition of cystine. Hence we conclude that Cys^{34}-blocked albumin which is commonly present in isolated albumin samples at a level of 30–50% of the albumin concentration is likely to be an artifact of the isolation procedure. This level of blocked Cys^{34} in isolated commercial albumin is evident from the spectrum shown in Fig. 1A where the n:n′ peak ratio is ca. 0.4 (blocked):0.6 (unblocked).

As glutathione disulfide did not react with albumin in blood plasma it seems unlikely that high levels of glutathione-blocked albumin occur in normal plasma, and we have not detected this derivative in isolated albumin by NMR [13,21]. Indeed the level of glutathione-blocking in bovine albumin reported by Andersson [22] is < ca. 10% of that of Cys-blocking. Also King has reported (briefly) that reactions of human albumin with glutathione do not go to completion [23]. Interactions between ionic charges may control entry into the Cys^{34} crevice, in particular reactions with negatively-charged substrates appear to be less facile, for example we find with isolated human albumin that iodoacetate reacts much less readily than does iodoacetamide, and that aurothiomalate does not react at all at low ionic strength (Christodoulou, J., Sadler, P.J. and Tucker, A. unpublished).

The anti-hypertensive drug captopril reduced Cys-blocked albumin which had been artificially generated in blood plasma by prior reaction with cystine and reversed the structural transition detectable via His^3. Keire et al. [24] have reported that captopril binds to lactate sites on albumin in plasma but does not undergo redox reactions. This further supports our suggestion that little of the albumin in normal plasma is present as a mixed disulfide.

The pH dependence of the extent of reaction of auranofin with albumin in blood plasma can be explained by the partial protonation of Cys^{34} at low pH and the expected lower reactivity of Au(I) to towards thiols compared to thiolates [25]. This behavior is similar to that which we have observed previously for isolated albumin [21,26] and provides further evidence for the low pK_a associated with the thiol group of Cys^{34}.

The reaction of disulfiram with albumin in blood plasma as

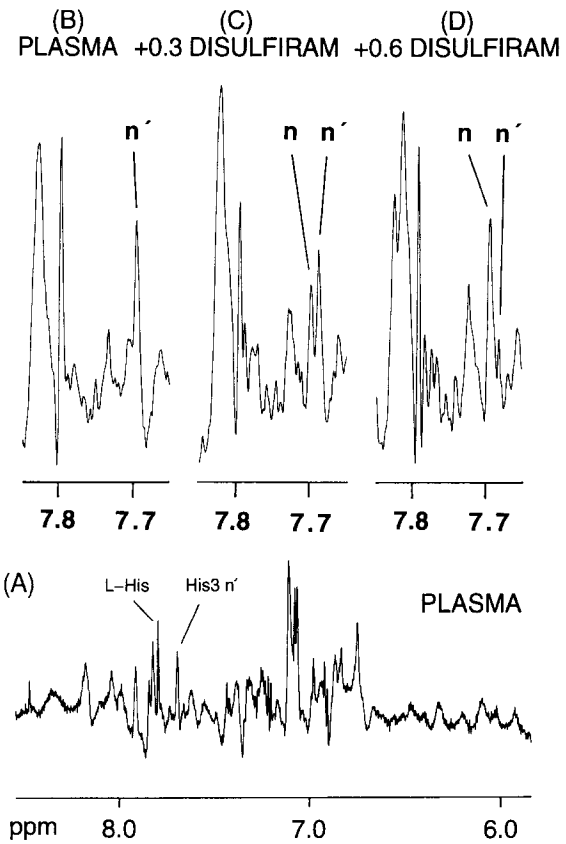


Fig. 4. 600 MHz ^{1}H NMR spectrum of (A) the aromatic region of fresh human blood plasma, together with expansions of the His^3 Hε1 region, (B) before, (C) after addition of ca. 0.3 and (D) a total of ca. 0.6 mol equiv of disulfiram (with respect to albumin). The only treatment to this sample involved the addition of a small amount of phosphate in D_2O as a lock and buffer (total 5% v/v, 10 mM phosphate). The H_2O peak was suppressed using the WATERGATE sequence [20]; if presaturation is used to suppress H_2O, then albumin also readily saturates by cross relaxation [19], although this effect can be used to aid assignment of low M_r (e.g. L-His) peaks.

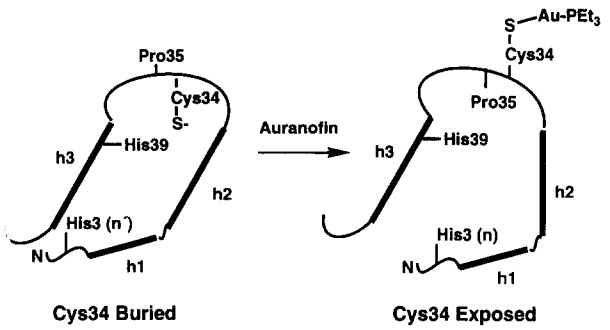


Fig. 5. Model for structural changes in domain IA of albumin. In the X-ray structure of human albumin with Cys^{34} in the thiolate form [4] Cys^{34} is in a crevice on the inside of a solvent-exposed unstructured loop. It is proposed that gold binding (shown), or oxidation to form a disulfide at Cys^{34}, or a sulfenic acid, leads to the movement of Cys^{34} from a buried to an exposed environment. This appears to be coupled to a movement of His^3 which is part of the N-terminal metal-binding site VI of albumin.

observed via the n′ → n switch confirms the postulated binding of disulfiram to Cys^{34} of albumin in plasma based on ^{14}C and ^{35}S labeling [27,28]. In addition the disulfide interchange at Cys^{34} explains the rapid disappearance of the administered disulfiram in vivo and appearance of its metabolite diethyldithiocarbamate.

Fig. 5 shows a model for the N-terminal region of albumin which is consistent with our observations. In the X-ray structure, Cys^{34} is situated on a loop of about 4 amino acids between helices h2 and h3 in domain IA and is protected from solvent in a crevice [5]. We propose that gold binding to Cys^{34} or oxidation to a disulfide is coupled to a structural change in the protein which is sensed by His^3. This might occur via *cis-trans* isomerization of Pro^{35} and movement of the intervening helices. We noted previously [21] for isolated albumin that the PEt_3 resonances of Alb-'Cys^{34}'-S-Au-PEt_3 are well-resolved, showing that this group is highly mobile on the protein and therefore likely to be in an exposed rather than in a buried environment. If these reactions involve a Cys^{34} 'flip-out', then they may involve large activation energies. Indeed Roberts et al. [29] have reported that the reaction of auranofin with bovine albumin is relatively slow ($k_1 = 2.94 \times 10^{-2}\ s^{-1}$). It is first order with respect to albumin but zero order with respect to auranofin, consistent with crevice-opening and exposure of Cys^{34} being the rate-limiting process.

Our observations help to explain the plethora of observations on albumin heterogeneity. The presence of low amounts of Cys^{34}-blocked albumin in fresh plasma is consistent with 5,5′-thiobis(2-nitrobenzoic acid) titrations of fresh blood plasma which show that the total thiol level is similar to the concentration of albumin [30], whereas the SH content of plasma stored for up to 6 weeks at −80°C [31] and isolated albumin [22] are typically only ca. 50–70% of the albumin level. The NMR method described here has the advantage of allowing direct and specific monitoring of structural changes at Cys^{34} of albumin in plasma. In isolated albumin the major crevice-opened forms are likely to be the Cys-blocked disulfide and Alb-'Cys^{34}'-SO_xH [32,18]. The disulfide but not all the sulfenic acid forms are readily reduced to the free thiolate with added thiols, a behavior analogous to that of Cys^{25} of papain [33]. It is likely that there are mechanisms for maintaining Cys^{34} of albumin in the free thiolate form in blood.

Acknowledgements: This work was supported by the Biotechnology and Biological Sciences Research Council, Engineering and Physical Sciences Research Council, and Delta Biotechnology Ltd (Nottingham). We thank the University of London Intercollegiate Research Service (NMR Centres at Birkbeck and Queen Mary and Westfield Colleges) and the Medical Research Council (Biomedical NMR Centre, Mill Hill) for provision of NMR facilities, Drs T. Peters Jr. and R. Reed (Cooperstown), Drs J. Woodrow and A. Quirk (Nottingham), Professor F.W. Putnam (Bloomington), Professor C.-B. Laurell (Malmö) and Professor H.A.O. Hill (Oxford) for stimulating discussions, suggestions and helpful comments.

References

[1] Peters Jr., T. (1975) in The Plasma Proteins (F.W. Putnam, Ed.) pp. 133–181, Academic Press, New York.
[2] Peters Jr., T. (1985) Adv. Prot. Chem. 37, 161–245.
[3] Kragh-Hansen, U. (1990) Danish. Med. Bull. 37, 57–84.
[4] Carter, D.C. and He, X.-M. (1992) Nature 358, 209–215.
[5] Carter, D.C. and Ho, X. (1994) Adv. Prot. Chem. 45, 153–203.
[6] Sadler, P.J., Tucker, A. and Viles, J.H. (1994) Eur. J. Biochem. 220, 193–200.
[7] Laussac, J. and Sarkar, B. (1984) Biochemistry 23, 2832–2838.
[8] Forster, J.F. (1977) in: Albumin Structure, Function and Uses (V.M. Rosenoer, M. Oratz and M.A. Rothschild, eds.), pp. 53–85, Pergamon, Oxford.
[9] Lewis, S.D., Misra, D.C. and Shafer, J.A. (1980) Biochemistry 19, 6129–6137.
[10] Pedersen, A.O. and Jacobsen, J. (1980) Eur. J. Biochem. 106, 291–295.
[11] Shaw, III. C.F., Isab, A.A. and Hoeschele, J.D. (1994) J. Am. Chem. Soc. 116, 2254–2260.
[12] Bos, O.J.M., Labro, J.F.A., Fischer, M.J.E., Wilting, J. and Janssen, L.H.M. (1989) J. Biol. Chem. 264, 953–959.
[13] Sadler, P.J. and Tucker, A. (1992) Eur. J. Biochem. 205, 631–643.
[14] Sadler, P.J. and Tucker, A. (1993) Eur. J. Biochem. 212, 811–817.
[15] Patel, S.U., Sadler, P.J., Tucker, A. and Viles, J.H. (1993) J. Am. Chem. Soc. 115, 9285–9286.
[16] Viles, J.H. (1994) PhD Thesis, University of London.
[17] Furst, D.E. and Dromgoole, S.H. (1984) Clin. Rheumatol. 3, 17–24.
[18] Shaw, C.F., III, (1989) Comments Inorg. Chem. 8, 233–267.
[19] Rabenstein, D.L., Mills, K.K. and Strauss, E.J. (1988) Anal. Chem. 60, 1380A–1391A.
[20] Piotto, M., Saudek, V. and Skenár, V. (1992) J. Biomol. NMR 2, 661–665.
[21] Cristodoulou, J., Sadler, P.J. and Tucker, A. (1994) Eur. J. Biochem. 225, 363–368.
[22] Andersson, L.-O. (1966) Biochim. Biophys Acta 117, 115–133.
[23] King, T.P. (1961) J. Biol. Chem. 236, PC5.
[24] Keire, D.A., Mariappan, S.V.S., Peng, J. and Rabenstein, D.A. (1993) Biochem. Pharmacol. 46, 1059–1069.
[25] Isab, A.A. and Sadler, P.J. (1982) JCS Dalton 135–141.
[26] i Dhubhghaill, O.M., Sadler, P.J. and Tucker, A. (1992) J. Am. Chem. Soc. 114, 1117–1118.
[27] Strömme, J.H. (1965) Biochem. Pharmacol. 14, 381–391.
[28] Johansson, B. (1990) J. Pharm. Pharmacol. 42, 806–807.
[29] Roberts, J.R., Xiao, J., Schliesman, B., Parsons, D.J. and Shaw, C.F. III (1994) Third Int. Conf. Gold and Silver in Med., Aug 18–20, Milwaukee, USA, Abs. P4.
[30] Ellman, G. and Lysko, H. (1979) Anal. Biochem. 93, 98–102.
[31] Chawla, R.K., Lewis, F.W., Kutner, M.H., Bate, D.M., Roy, R.G.B. and Rudman, D. (1984) Gastroenterology 87, 770–776.
[32] Coffer, M.T., Shaw, C.F. III, Eidsness, M.K., Watkins, J.W. II and Elder, R.C. (1986) Inorg. Chem. 25, 333–339.
[33] McGarth, M.E., Wilkie, M.E., Higaki, J.N., Craik, C.S. and Fletterick, R.J. (1989) Biochemistry 28, 9264–9270.