Some effects of post-translational N-terminal acetylation of the human embryonic ζ globin protein

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Using site-directed mutagenesis we have produced the first mutant form of a human embryonic haemoglobin. We have mutated the N-terminal Ser residue of the ζ-chain of haemoglobin Portland, $\zeta_2\gamma_2$, (which is normally acetylated) to a Val (which possesses a free amine terminus). The protein spontaneously assembles into a fully functional tetramer which shows co-operative oxygen binding. Determination of the reactivity of the mutant protein with 2,3-diphosphoglycerate indicates that the mutation process does not lead to any major disruption of the protein structure. A comparison of the properties of the mutant and wild-type proteins identifies a significant role for the normal N-terminal acetylation of the ζ-chain with regard to the alkaline Bohr effect and the sensitivity of the oxygen affinity of the protein towards chloride ions. The possible physiological significance of this modification is discussed.

INTRODUCTION

Only within the past year have extensive oxygen-binding data become available for the three human embryonic haemoglobins [1]. Within this group of oxygen-binding proteins a globin chain occurs which is expressed only at the earliest stages of development, namely the ζ globin. The ζ globin chain is unique amongst the human globins in that it is the only globin protein found to be totally N-terminally modified [2,3]. The fetal γ-chain is reported to be only 15% N-terminally modified *in vivo* [4]. Over the years the role of the N-terminus of the α globin chain has been widely debated. The N-terminus of the α-chain has been shown to play an active role in the modulation of haemoglobin function, particularly in the chloride-dependent Bohr effect, contributing up to 25% of the alkaline Bohr effect [5–7]. Weak inorganic anion-binding to the N-terminal Val of the α-chain was suggested some years ago but recent X-ray diffraction studies have failed to support these earlier findings [8–10]. Although the N-α-terminus clearly has a part to play in the modulation, by chloride ions, of the binding of oxygen, the mechanism whereby this is achieved has only recently been formulated. Most of the chloride sensitivity of the adult protein has been correlated with the existence of a cationic excess within the central water-filled cavity which is neutralized by the random binding of chloride ions [11–13]. Within this scheme, the role of Val-1 α is envisaged as being somewhat different. It lies not within the cavity but at one edge [14,15], and its contribution may well arise not from simple electrostatic contributions but via an entropic effect manifest in its significant degree of mobility in the oxygenated form of the protein [11,16].

The three human embryonic haemoglobins exhibit intrinsic oxygen affinities very similar to that of the adult protein [17]. However, they lack chloride-ion sensitivity and this appears to be the major factor which provides the affinity gradient necessary for oxygen transfer from the mother to the embryo under physiological conditions. The situation pertaining in the embryo thus closely mimics that at the fetal stage of development where lack of organic phosphate sensitivity provides the affinity gradient necessary for placental oxygen transfer. With the recent advent of plasmid-based eukaryotic expression systems for native haemoglobins containing ζ-chains [1,18], the opportunity now arises to allow us to make an investigation of the role N-terminal acetylation plays in controlling the oxygen-binding properties of the ζ-chains. To this end we have compared the properties of recombinant human embryonic haemoglobin Portland ($\zeta_2\gamma_2$), which contains N-terminally acetylated ζ-chains, with those of a genetically engineered, full-length, mutant form which possesses a free N-terminal amino acid on the ζ-chain. During the course of this study we also produced the $\zeta_2\epsilon_2$ protein. However we do not present any data on this system as the mutant $\zeta_2\epsilon_2$ form is very unstable and has a very high oxygen affinity, such that accurate quantitative comparisons are not possible. The results obtained from the two $\zeta_2\gamma_2$ systems allow us to identify the role played by the naturally occurring post-translational modification and furthermore clarify the role of the free N-α-terminus in the other globin proteins.

MATERIALS AND METHODS

Human embryonic haemoglobin Portland ($\zeta_2\gamma_2$) was produced in a yeast expression system as previously described [19]. The mutant form of this protein was produced by oligonucleotide-directed mutation of the Portland expression plasmid. The naturally occurring N-terminal Ser residue of the ζ-chain, which is normally post-translationaly acetylated, was mutated into a Val residue, using a 27mer oligonucleotide primer containing two mis-matched bases, employing standard protocols and a commercially available mutagenesis system (Amersham, U.K.).

Both native and mutant proteins were purified by employing the procedures reported previously [1,18]. Globin chain separation, HPLC, amino acid sequencing and M_r determination of the holoproteins were performed as described earlier [18]. Accurate M_r determinations were made using electrospray mass spectrometry (ES-MS) employing a VG Quattro II spectrometer (VG Biotech, Cheshire, U.K.) [20,21].

Oxygen-binding curves were obtained using either a HEMOX

Abbreviations used: 2,3-DPG, 2,3-diphosphoglycerate; ES-MS, electrospray MS.
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oxygen dissociation analyser [22] or a system constructed in this laboratory[17].

RESULTS

Expression of the mutant haemoglobin Portland [ζ_2 (Ser-1NAc→Val)γ_2] in the yeast expression system, previously described [19], yielded approximately 70 mg of haemoglobin per litre of culture. The protein was purified as described and characterized in order to verify the nature of the expression product. When subjected to HPLC separation the mutant protein gave two clean peaks, indicative of a very high degree of purity (Figure 1). One of the peaks, corresponding to the γ-chain, eluted at a position identical with that observed in the chromatograph of the wild-type $\zeta_2\gamma_2$ protein. The peak corresponding to the mutant ζ-chain eluted somewhat earlier than the corresponding N-terminally acetylated wild-type ζ-chain. Each of the protein peaks obtained by HPLC of the mutant protein was subjected to ten cycles of N-terminal amino acid sequencing in the gas-phase sequencer. The γ-chain showed the sequence expected for the wild type. The ζ-hain however showed the presence of an unblocked N-Terminal Val residue followed by the predicted amino acid sequence, in contrast to the wild-type ζ-chain which has been shown by fast atom bombardment MS of tryptic digests to retain an acetylated N-terminal Ser residue [1]. The ratio of the intensity of the haem peak to the protein peaks obtained from the HPLC traces also indicates that the mutant protein contains the normal complement of haem groups.

To eliminate the possible occurrence of unpredicted alterations in the amino acid composition of the mutant protein we subjected the holoprotein to ES-MS (Figure 2). The wild-type $\zeta_2\gamma_2$ gave mass values of 16008.9 for the γ-chain (compared with the theoretical value of 16009.3) and 15547.6 for the ζ-chain (compared with 15547.9 calculated for the N-terminally acetylated chain). The mutant form of the protein gave mass values of

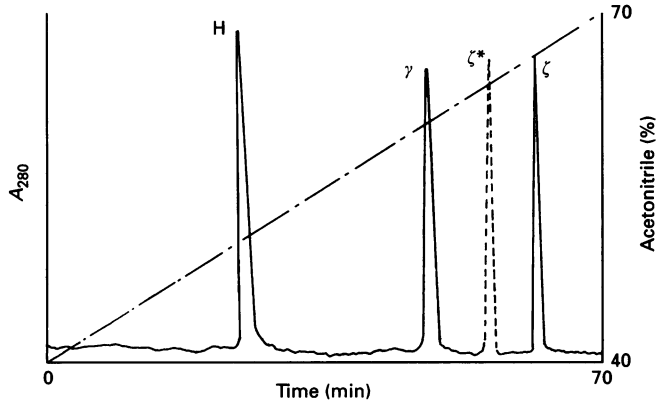

Figure 1 Globin separation by HPLC

Samples of the wild-type and mutant human embryonic haemoglobin Portland ($\zeta_2\gamma_2$) were separated by reverse-phase HPLC on a C_8 column. The samples were dissolved in 2.5% trifluoroacetic acid and the column was developed with a gradient from 40 to 70% acetonitrile (diagonal broken line). Peak H represents haem. The figure shows an overlay of the chromatographs for the wild-type $\zeta_2\gamma_2$ and that for the mutant ζ_2(Ser-1NAc→Val)γ_2, indicating the elution positions for the wild-type γ- and ζ-chains and the mutant ζ^*-chain.

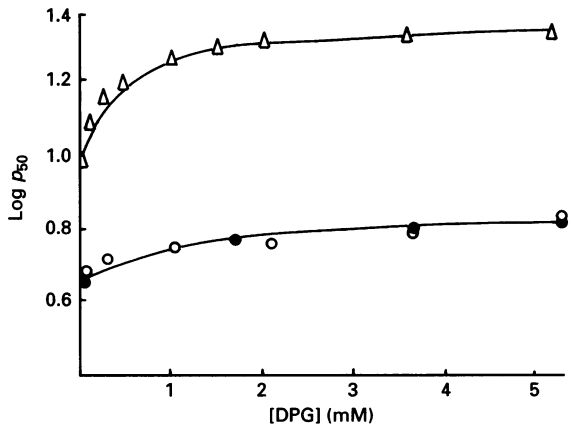

Figure 3 The effect of 2,3-DPG concentration on the binding of oxygen to haemoglobin Portland ($\zeta_2\gamma_2$)

Oxygen-binding curves were determined for wild-type (○) and mutant (●) forms of haemoglobin Portland in 50 mM Bistris buffer at pH 7.4 and 37 °C in the presence of varying concentrations of 2,3-DPG. The curve for the adult protein is included for comparison (△).

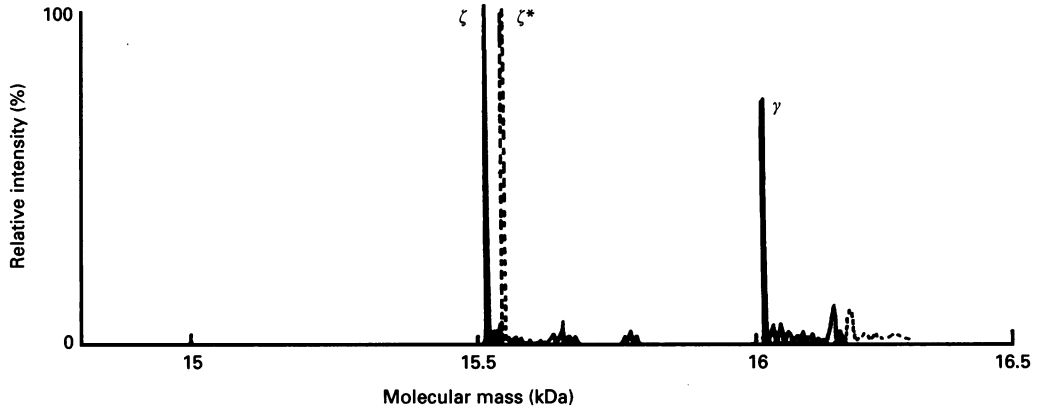

Figure 2 Maximum entropy transformed ES-MS

The maximum entropy transform of the ES-MS of the wild-type (continuous line) and mutant form (broken line) of $\zeta_2\gamma_2$ are shown overlaid. The relative positions of the wild-type γ- and ζ-chains are indicated together with that of the mutant ζ^*-chain.

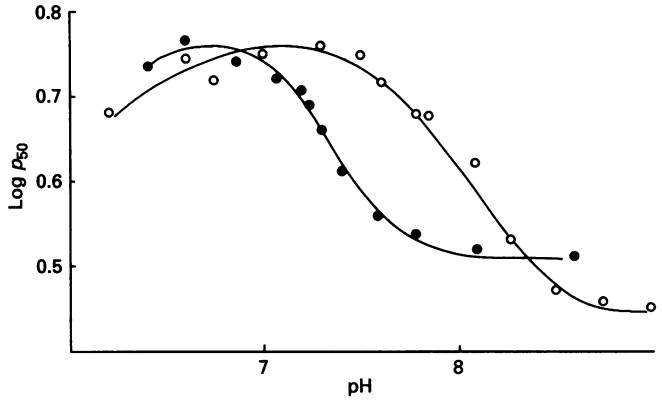

Figure 4 The Bohr effect of wild-type and mutant haemoglobin Portland

Oxygen-binding curves were measured in 50 mM Bistris buffer containing 100 mM sodium chloride at 37 °C for the wild-type (○) and mutant (●) forms of haemoglobin Portland.

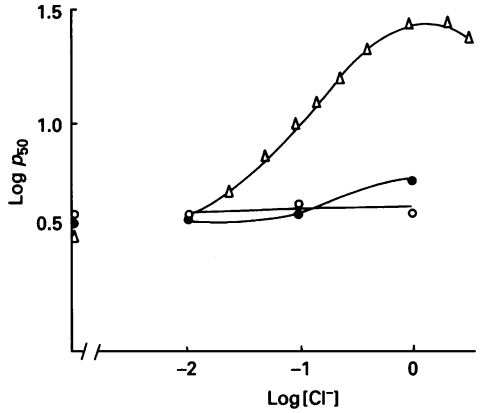

Figure 5 The effect of chloride ions on oxygen binding to human haemoglobin Portland

Oxygen-binding curves were measured at 37 °C in 50 mM Hepes buffer at pH 7.4. Chloride-ion concentration was altered by the addition of a concentrated solution of sodium chloride. The curves are shown for the wild-type (○) and mutant (●) forms of haemoglobin Portland. The curve obtained for the adult protein (△) is shown for comparison. The points on the *y*-axis correspond to the values obtained in the absence of chloride ions.

16009 for the γ-chain and 15517.9 for the ζ-chain (compared with a theoretical value for the free N-terminal Val form of the protein of 15517.95). Very minor components were also seen in ES-MS, corresponding to the presence of normal protohaem IX still associated with the protein chains.

The allosteric characteristics of the mutant protein have been studied with regard to its interactions with organic phosphates, protons and chloride ions. When the oxygen affinity of the mutant protein was compared with that of the wild type in the presence of various concentrations of 2,3-diphosphoglycerate (2,3-DPG) it was found that its sensitivity towards this organic phosphate allosteric effector, although quite different from that of the adult protein, was, within experimental error, indistinguishable from that of the wild-type embryonic protein (Figure 3). In response to variations in solution pH, the mutant protein exhibits a slightly steeper and somewhat shifted dependence of oxygen affinity on pH, as compared with that of the wild-type protein (Figure 4). At a fixed pH of 7.4 the wild-type and mutant

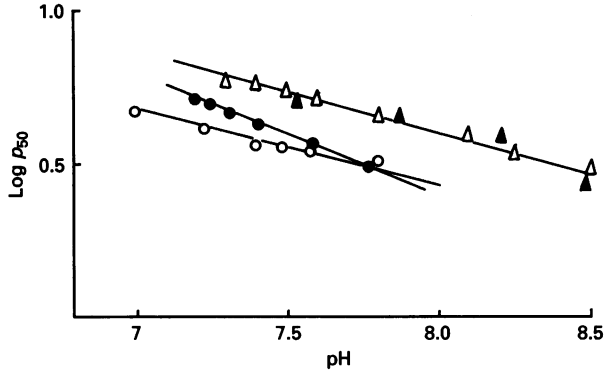

Figure 6 The effect of chloride ions on the alkaline Bohr effect of haemoglobin Portland

The Bohr effect is shown for the wild-type (△, ▲) and mutant (○, ●) forms of haemoglobin Portland. The open symbols represent data obtained in the absence of chloride ions and the filled symbols data obtained in the presence of 100 mM sodium chloride.

forms of the protein show small but significant differences in their sensitivity of oxygen affinity towards chloride-ion concentration (Figure 5). The contribution that the chloride-dependent component of the Bohr effect makes to the overall pH sensitivity of the proteins can be judged by a comparison of the alkaline Bohr effects in the presence and absence of chloride ions (Figure 6). The magnitude of the alkaline Bohr effect, expressed as $\Delta\log p_{50}/\Delta\log$ pH (where p_{50} is the oxygen partial pressure necessary to achieve 50% saturation of the haemoglobin), is −0.26 for the wild-type $\zeta_2\gamma_2$ protein, independent of the chloride-ion concentration, whilst that of the mutant varies from −0.24, in the absence of chloride ions, to −0.34 in the presence of 100 mM chloride ions (Figure 6).

DISCUSSION

The results presented above show that using the yeast-based plasmid expression system for haemoglobin Portland ($\zeta_2\gamma_2$) it is possible to produce a fully functional N-terminally mutated form of the protein possessing a free N-terminal amine group on the ζ-chain. A comparison of the wild-type and mutant forms of the protein then allows us to identify the role played by the naturally occurring ζ-chain N-terminal acetylation. Under 'standard physiological' conditions both the wild-type and mutant proteins exhibit high affinity and a relatively high degree of co-operativity, with the mutant form displaying a slightly lower oxygen affinity. When studying site-directed, mutated proteins it is important to be sure that no major structural change has occurred consequent to the mutation. Although this is best achieved by a comparison of the full three-dimensional structures of the proteins, these are not yet available, so we have taken a functional approach. It is well recognized that the binding of 2,3-DPG occurs between the β-type chains of the deoxy form of haemoglobin, at a site far removed from the site of mutation used in this study. Furthermore the binding of 2,3-DPG is known to be sensitive to changes in the structure of the binding site [23,24]. When we compare the binding of 2,3-DPG with the wild-type and mutated forms of the proteins (Figure 3) we find that their binding characteristics are indistinguishable and we take this as evidence that no major structural changes have occurred consequent to the mutation of the N-terminal residue of the ζ-chain of the protein.

The N-terminal residue of the α-chain has been identified as making a significant contribution to the chloride-dependent

Bohr effect [5–7]. This finding is further substantiated by a comparison of the alkaline Bohr effects of the wild-type and mutated forms of haemoglobin Portland made in the presence of 100 mM sodium chloride (Figure 4). A quantification of the effect of chloride ions can be made in two ways. At a fixed pH of 7.4, the oxygen affinity of the wild-type protein shows no sensitivity towards the presence of chloride ions. The mutant form shows a small but significant sensitivity towards chloride ions with an associated value of $\Delta\log p_{50}/\Delta\log [Cl^-]$ of 0.14 compared with a value of 0.45 exhibited by the adult protein (Figure 5). The effect of the interaction of chloride with the protein on the Bohr effect is also clearly different for the two forms of haemoglobin Portland. When the Bohr effect is quantified over the range pH 7.0–8.5 (Figure 6) the lack of chloride sensitivity of the wild-type form of the protein is evidenced by an identical value of $\Delta\log p_{50}/\Delta\log$ pH of -0.26, both in the presence and absence of 100 mM sodium chloride. In the case of the N-terminally mutated form a different situation arises. In the absence of chloride ions the value of $\Delta\log p_{50}/\Delta\log$ pH is -0.24 but becomes -0.34 in the presence of 100 mM sodium chloride. This compares with values of -0.25 and -0.45 respectively for the adult form of the protein [17]. These data are thus consistent with previous findings employing naturally occurring mutants of the adult protein, which suggest that the N-terminal amine group of the α-chain contributes to the chloride-concentration-dependent Bohr effect, making a contribution of approximately 25% to the overall process [5–7].

From the above it is clear that the N-terminal amine group of the ζ-chain has the capacity to produce within the haemoglobin Portland molecule a chloride-dependent Bohr effect of almost identical magnitude with that of the non-mutated form. It has recently been found that the absence of chloride sensitivity is predominantly responsible for providing the affinity gradient necessary for oxygen scavenging from the maternal system by the embryonic haemoglobins [17]. The N-terminal acetylation of the ζ-chain thus has a significant role to play in the depression of the chloride-ion sensitivity of haemoglobin Portland and the provision of adequate oxygen supplies to the developing embryo. It is also interesting to note that as the embryo develops and moves into the fetal stage of development, when placental oxygen transport requires fetal haemoglobins of a somewhat lower affinity than the embryonic forms, there is an accompanying shift from ζ-chain-containing systems to an α-chain-based system with a free N-terminal amino acid which exhibits a larger chloride effect.

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