Reactivity and Ionization Constants of the Lysine Residues in Apovitellenin I of Emu Egg Yolk Low-density Lipoprotein by Competitive Labelling

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Abstract
Competitive labelling with $[^{14}C]$acetic anhydride over a range of pH values has been used to explore the surface topography of the apovitellenin I moiety in emu egg yolk low-density lipoprotein. The reaction of the lysine e-amino groups with acetic anhydride has been related to pH in a set of titration curves; from these, the reactivities relative to alanine and the ionization constants of all but the amino terminal lysines have been determined.

All lysines have near normal $pK_a$ values around 10, and lower than normal reactivities (except the amino terminal lysine).

At pH values above 10, the titration curves show breaks where the e-amino groups become much more reactive, except for lysine 71 which in this regard behaves like a normally ionizing lysine in not showing a discontinuity.

Most of the basic residues in this apoprotein may occur clustered at the surface of the molecule. This accounts best for the observed low reactivities and $pK_a$ values. The amino terminal lysine residue is presumably completely exposed to the aqueous environment.

Introduction
Kaplan and his associates (Kaplan et al. 1971) have shown that the method of competitive labelling with electrophilic reagents in the presence of a standard nucleophile is a valid and valuable method to determine $pK$ values of nucleophilic residues in native proteins. The results obtained for elastase (Kaplan et al. 1971), chymotrypsin (Kaplan 1972) and calf thymus histones (Malchy and Kaplan 1974) agree well with results obtained from other evidence. This paper describes an attempt to use the principle of competitive labelling in order to determine the role of the lysine residues in apovitellenin I from emu egg yolk low-density lipoprotein in lipid binding. The understanding of such interactions is basic to the understanding of membrane structure.

Materials and Methods
Low-density lipoprotein from emu (Dromaius novaehollandiae) egg yolk was a gift from Dr R. W. Burley. Trypsin (TPCK-treated) came from Worthington Biochemical Corp. (Minnesota), while thermolysin was from Calbiochem (La Jolla, California). $[^{14}C]$Acetic anhydride (c. 125 mCi/mmol) came from the Radiochemical Centre, Amersham (England). For all paper separations, Whatman 3MM paper was used.

Acetylation of Lipoprotein
Aliquots (1·5 ml) of 16% lipoprotein solution (containing c. 15 mg apovitellenin I) in water were dialysed at 4°C for 48 h against three changes of buffer. These buffers were prepared from a
universal buffer containing phosphoric acid (0·04M), acetic acid (0·04M) and boric acid (0·04M); aliquots of this universal buffer were titrated to the desired pH with 0·2M NaOH, as measured at 25°C (Britton and Robinson 1931). Buffers of the following pH values were used: 8, 8·5, 9·0, 9·4, 9·8, 10·2, 10·6 and 11·0. At the conclusion of the dialysis, each sample was adjusted to 2·0 ml with the appropriate buffer, and to each sample was added 2·0 μmol l-alanine in 100 μl water; the samples, in stoppered tubes, were stirred continuously at 25°C for 10 min. At that stage, 50 μl dry acetonitrile, containing 0·33 μmol 1[^14]C]acetic anhydride (41·2 μCi) was added with stirring, and the reaction mixture was allowed to stand at 25°C for 2 h. The samples were then dialysed individually against 200 ml water at 4°C overnight; these dialysates were processed to recover acetylated alanine, as described later. After further dialysis for 24 h against 6M urea–0·05M HCl, lipids were removed from the samples and the apovitellin I fractions prepared as described by Burley (1973). The purified apoprotein fractions were dialysed against water, then completely acetylated with five 25-μl aliquots of acetic anhydride at pH 8 (pH-stat, 5M NaOH); finally they were dialysed overnight against 0·1M N-ethylmorpholine acetate, pH 8·0.


The dialysed protein fractions were digested overnight at 39°C with trypsin (0·5 mg), then with thermolysin (0·5 mg) for 2 h. After freeze-drying, the samples were purified in this sequence: (1) high-voltage paper electrophoresis at pH 3·5, (2) paper chromatography in n-butanol–acetic acid–water–pyridine (15 : 3 : 12 : 10 v/v) and (3) high-voltage paper electrophoresis at pH 1·9. The purification was monitored with ninhydrin and by autoradiography. This regime gave pure peptides, which included every lysine residue in the sequence.

**Purification of N-[^14]C]Acetylated Alanine**

The aqueous dialysate resulting from the first dialysis of the labelled protein was rotary evaporated and the residue acetylated with 200 μl acetic anhydride and 50 μl pyridine for 72 h. After drying in vacuum the samples were redissolved in 1 ml water, acidified to pH 1 and extracted with six 3-ml aliquots of ethylacetate; the extracts were evaporated to dryness in a nitrogen stream.

Paper chromatography of the residues in butanol–acetic acid–water–pyridine (15 : 3 : 12 : 10 v/v), followed by high-voltage paper electrophoresis at pH 6·5 gave pure samples of N-[^14]C]acetylated alanine. The purification was monitored by autoradiography.

**Amino Acid Analysis**

Amino acids were analysed on a Beckman 120C analyser, using a single column program. Peptides were hydrolysed in 200 μl constant boiling HCl, in vacuum, for 24 h at 110°C.

**Scintillation Counting**

Samples were counted on a Packard Tri-Carb 500 C liquid scintillation counter, using dioxan containing naphthalene (120 g/l), 2,5-diphenyloxazole (4 g/l) and 1,4-bis-(5-phenyloxazol-2-yl)-benzene (50 mg/l) as the scintillation fluid.

**Results**

The specific radioactivities of the acetylated standard nucleophile alanine (RS) and of the acetylated lysine peptides (RA) are related by the following expression (Kaplan et al. 1971):

\[ \alpha_A r = \frac{\alpha_S \times \text{specific radioactivity of } RA}{\text{specific radioactivity of } RS}, \]

where

\[ \alpha_A = 1/[1+(H^+)/K_a], \quad \alpha_S = 1/[1+(H^+)/K_s], \]

\( \alpha_A \) is the degree of ionization of the standard nucleophile, \( \alpha_S \) is the degree of ionization of the residue investigated, \( R \) is the radioactive electrophilic reagent, \( r \) is the ratio between the rates of acetylation of \( A \) and \( S \), and \( K_a \) and \( K_s \) are acid dissociation constants. \( pK_s \) was taken as 9·87 at 25°C (Dawson et al. 1969).
The specific radioactivities of each peptide except the peptide containing lysine 1 were used to calculate $a_{\varphi}$ over a range of pH values. No calculations were made for lysine 1; it is the amino terminal residue of the apoprotein, and since it carries a free $\alpha$-amino group as well as an $\varepsilon$-amino group, no unambiguous assignment of label be made. However, the specific radioactivity of the dipeptide (Ac)$_2$-Lys-Ser at pH 8·0 and 8·5 was very close to twice (1·95 and 2·10 respectively) the specific activity of the standard N-acetyllalanine at these pH values.

Fig. 1 shows that data obtained for lysine residues 29, 33, 60, 73 and 79 can be fitted, at least at the lower pH values, to titration curves for groups with $pK_a$ values close to 10 but with reactivities, in relation to the free nucleophile, rather lower than unity. Above pH 10 the observed titration behaviour departs from the calculated curves, indicating major changes in the environment of the $\varepsilon$-amino groups. Lysine 71, however, closely adheres to the values calculated and has a reactivity close to unity.

**Discussion**

The understanding of lipid–protein interaction is essential to the understanding of membrane structure, and yolk lipoproteins provide a relatively simple system, both in composition and availability, in which to study such interactions.
Gotto et al. (1970) proposed, on the basis of spin label experiments, that some lysine ε-amino groups in both high- and low-density serum lipoproteins were directed into hydrophobic areas. An examination of the sequence of the main apoprotein of low-density emu egg yolk lipoprotein (Dopheide and Inglis 1974) shows areas which by the usual conventions (Capaldi and Vanderkooi 1972) can be classed as hydrophobic (residues 12–24, 37–48) and also a very polar amino terminal sequence (1–11). This might indicate domain formation, tending to polarize the protein molecule when interacting with lipid.

This study on the accessibility of the lysine residues to acetic anhydride by the method of competitive labelling (Kaplan et al. 1971) indicates that in the yolk protein not all lysine residues are equivalent. Titration curves were obtained for all but the amino terminal lysines in the molecule; at the lower pH values these curves fit calculated data for ε-amino groups with a pK value near 10, which is a little below normal at 25°C (Ellenbogen 1952). Since the pK values of the titrated groups are close to 10, and the pK value of the standard nucleophile, alanine, is also close to 10 (i.e. 9.87 at 25°C) (Dawson et al. 1969), the reactivity of the lysines can be expressed quantitatively in terms of the reactivity of alanine. The r values then give an approximate measure of ‘buriedness’. From a Bronstedt plot for the reaction of acetic anhydride with primary amines, Kaplan et al. (1971) defined the reactivities of the two lysine residues in porcine elastase as 1.3 and 1.6; these residues had normal pK_a values. Kaplan et al. (1971) concluded from this that the elastase lysines are situated at the surface of the molecule and are freely accessible to the acylating agent. They point out that pK_a is a property of the group and its environment, while reactivity reflects steric hindrance. A reactivity much lower than unity indicates considerable hindrance; this is well illustrated for the amino terminal value of elastase which has the high pK_a value of 9.7 (the effect of ion-pair formation) and an almost negligible reactivity [the effect of burial, demonstrable in the three-dimensional X-ray model (Shotton and Watson 1970)]. The reactivities of all lysine side-chains in the apoprotein, except that of the amino terminal residue, has been found to be less than unity. Thus, lysines 29, 33, 60 and 73 are of low reactivity, lysine 71 is close to normal reactivity, while lysine 79 is in an intermediate position. Lysine 71 also titrates normally over the range investigated; the others all show a break in the titration curve around pH 10, indicating a change of environment with a consequent increase in reactivity. The amino terminal lysine residue appears to be fully exposed to the aqueous solvent. Although the incorporation of radioactivity in it cannot be assigned separately to either the ε- or the ε-amino groups, at pH 8 and 8.5 the specific activity is nearly double that of the alanine standard. This is consistent with the hydrophilic character of the amino terminal sequence of the protein, which is expected to be directed into the aqueous environment.

In what way the ‘buried’ lysines are involved in lipid binding is still conjectural. Extended dialysis of the whole lipoprotein complex at pH 11 fails to dissociate any lipid from the complex, even though at that pH the ε-amino groups are substantially deprotonated. Hence they do not seem to play an important role in lipid binding, though in view of the lowered reactivities of the lysine ε-amino groups they appear to be involved in some type of interaction which decreases their reactivity; the decreased reactivity occurs without a rise in pK_a value, which argues against ion-pair formation stabilized by burial. Rather, pK_a values lowered from a normal 10.4
(Ellenbogen 1952) to near 10.0 may indicate a clustering of positive charges at the surface of the protein. This would tend to both depress $pK_a$ values and lower the reactivity of the $\varepsilon$-amino groups. An examination of the sequence of apovitellenin I (Dopheide and Inglis 1974) shows that all basic residues in this protein occur within four short 'stretches' totalling 34 amino acids out of a total chainlength of 84 residues; these basic stretches are separated by quite hydrophobic sequences.

Assmann and Brewer (1974) point out the possible importance for lipid binding of amphiphatic helical segments in the serum high-density apoproteins. However, an examination of emu apoprotein does not indicate sequences capable of forming appreciable helical segments of the type proposed by Assmann and Brewer (1974), so that this protein may interact with lipid in a manner different from that proposed for the serum high-density apoproteins.

References


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