THE USE OF SUBUNIT EXCHANGE CHROMATOGRAPHY FOR THE GROUP SPECIFIC FRACTIONATION OF HISTONES

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SUMMARY. Starting from the histone mixture obtained from calf thymus, the arginine rich fraction ARE+) was coupled to organomercurial agarose via a mercaptide bond to one of its cysteines. ARE-agarose proved to be useful for a large scale affinity chromatographic separation of whole histone. In 1M NaCl, pH 4.5, highly pure histone fractions could be eluted with an urea gradient revealing increasing affinity towards ARE in the order: KAP < KAS < LAK < $(ARE)_n(GRK)_n$ < GRK < ARE.

Considerable progress in the understanding of the biochemical role of the histones has recently been made by elaborating the concept that their close primary structure-function relationship is mainly reflected in the specificity of histone-histone interactions. Except for the lysine rich fraction KAP, the other four principal histones bind to DNA as well defined subunit complexes (2, 3)

Specific cross interactions between histones have been reported for LAK and KAS (4-6), KAS and GRK (4, 7) and LAK and GRK (6, 8), all obtained by classical extraction procedures under denaturing conditions. Of these, a role in situ has so far only been established for a complex $(LAK)_n(KAS)_n$, which could be displaced from chromatin together with a well defined tetramer, $(ARE)_2(GRK)_2$. At this point, a relevant question remains, whether functional histone complexes can, in principle, be reconstituted from denatured species, or if their generation requires additional elements, e. g. the DNA surface as a matrix.

+) The histone nomenclature follows recommendations quoted in ref. (1). Other abbreviations used are: DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid); pCMB, p-chloromercuribenzoate

Following the principle of "subunit exchange chromatography" elaborated by Antonini et al. (9) a possible "recognition site" for $(ARE)_n$ $(GRK)_n$ species (present in a previously denatured histone mixture) would be either one of its components. We chose fraction ARE for the following reasons:

a) The usual coupling to CNBr activated agarose would in the case of histones lead to heterogenous and/or multiple attachment as they contain a large number of lysines in their terminal segments. Calf thymus histone ARE offers cysteines in position 96 and 110 as alternative sites for fixation. Taking advantage of differential reactivities of these cysteines (10, 11) it should be feasible to bind ARE by an organomercurial spacer via a distinct SH group; this procedure would result in a homogenous matrix of histone molecules in their native conformation. A separation of whole histone prior to coupling is unnecessary because ARE is the only fraction to contain cysteine.

b) The concentration of organomercurial functions can easily be determined ; mercaptide bonds are cleavable allowing a routine control of the quality and quantity of coupled histone (12).

EXPERIMENTAL

Whole histone was isolated from freshly dissected calf thymus glands according to Kobayashi and Iwai (13) except that a saline solution 0.15 M NaCl, 0.05 M $NaHSO_3$ and 3 mM EDTA (pH 4) was used. This modification was introduced to prevent the sulfhydryl oxidation (14) and proteolytic degradation (15) found in commercial preparations. After homogenation with a Waring blender the suspension was sonicated (Branson sonifyer; 5 10 - second strokes for each 250 ml sample). Dialysis was performed against 1 mM EDTA containing 0.02 % sodium azide. Histones were lyophilized and stored below 0° C.

Sulfhydryl contents were analyzed with DTNB by the Ellman procedure(16).

Organomercurial agarose was prepared according to Cuatrecasas from aminoethyl agarose and pCMB (17). The ligand concentration was assayed by determining the cysteine binding capacity (cysteine concentrations were measured by DTNB titration) or turbidometrically (18) after displacement of coupled histone ARE by ß-mercaptoethanol (12).

Gel electrophoreses were performed according to Panyim and Chalkley (19) in 15 % acrylamide gels (6.25 M urea) and to Alfagene et al. (20) in 12 % acrylamide gels (6 M urea, 0.38 % Triton DF 16).

RESULTS AND DISCUSSION

Characterization of ARE-sulfhydryl groups

A sample of whole histone prepared under sulfhydryl preserving conditions (see experimental part) was subjected to a kinetic analysis with DTNB. The time course, analyzed by standard procedures (21-23) revealed a fast and a slow phase of identical amplitude, indicative for two classes of sulfhydryls with equal population. Moreover, the final absorption at 412 nm, A_∞, reached the value expected for a 20 % ARE content of whole histone (table 1).

Table 1: Analysis of histone ARE sulfhydryl reactivities.

5 mg whole histone was dissolved in 2 ml Na-phosphate buffer, pH 8. DTNB-solution (50μl) was added to a concentration of 2.5×10^{-4} M. k_f and k_s are the bimolecular rate constants for the fast and the slow phase.

Medium: Na-phosphate	fast amplitude / total amplitude	k_f (min^{-1})	k_s (M^{-1})	k_f/k_s	A_∞	SH-groups in 38000 d histones
0.1 M	0.50	1800	103	18	0.92	1.1
0.01 M	0.45	21000	670	31		

Model considerations based on esr evidence show Cys 96 to be buried in a region of high helix forming potential whereas Cys 110 seems to be more accessible as it is located outside the hydrophobic core (24). Therefore the coupling reaction should be directed towards position 110 by the choice of conditions likely to yield a high k_f/k_s ratio (table 1). A reaction with Cys 96 will be prevented if the nearest neighbor distance of pCMB ligands significantly exceeds the stretch spanned by the ARE segment 96-110; our data show this to be the case:

The ligand concentration of organomercurial agarose was determined to be 0.65 μ moles/ml corresponding to a 150 Å spacing between pCMB functions (if an even distribution within a cubic lattice is assumed). In case of a one cysteine attachment the binding capacity of this adsorbent would be 10 mg ARE/ml; the amount of ARE recovered after the removal of all noncovalently bound protein by 8 M urea (8.3 mg) is in reasonable agree-

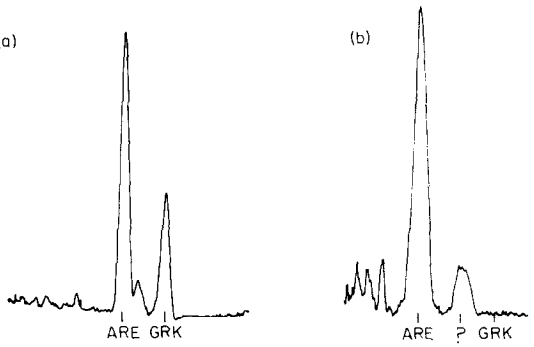

Fig. 1. Composition of histone, immobilized by pCMB agarose

25 ml sedimented pCMB-agarose were added to a solution of 2.5 g whole histone in 0.01 M Na-phosphate and filled up to 50 ml. The suspension was stirred at 4° C over night, then poured into a column (1.5×14.5 cm). Here the adsorbent was extensively (12 h) washed with

6 M urea, 0.05 M EDTA, 0.02 % NaN_3 (fig. 1a) or
8 M urea, 0.05 M EDTA, 0.02 % NaN_3 (fig. 1b) resp.

Histone remaining was removed from 1 ml samples of substituted agarose by boiling with 3 ml 0.01 M Na-phosphate, 5 % ß-mercaptoethanol (pH 8) and analyzed by gel electrophoresis

on regular gels (ref. (19), fig. 1a) and
in presence of 0.38 % Triton DF 16 (ref. (20), fig. 1b) resp.

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ment with this prediction. Values determined after washing with 6 M urea were consistently higher (12-15 mg/ml) and this result is explained by tight association of solute histone GRK (fig. 1 a) and probably ARE with matrix bound ARE.

Fractionation of whole histone on ARE-agarose

A urea gradient between 0 and 8 M leads to the elution of four main bands (I-IV) from ARE-agarose (fig. 2) which were analyzed by gel electrophoresis under two different sets of conditions to detect and quantitate all species contributing to the over all absorption (fig. 3,4).

Histone KAP was eluted directly after the dead volume of the column forming a symmetrical band (I) as expected for a non crossinteracting species. According to conventional gel electrophoresis peak II could consist of either KAS or LAK or both (fig. 4, trace b_1). It is well known however that Triton detergents retard LAK more strongly than

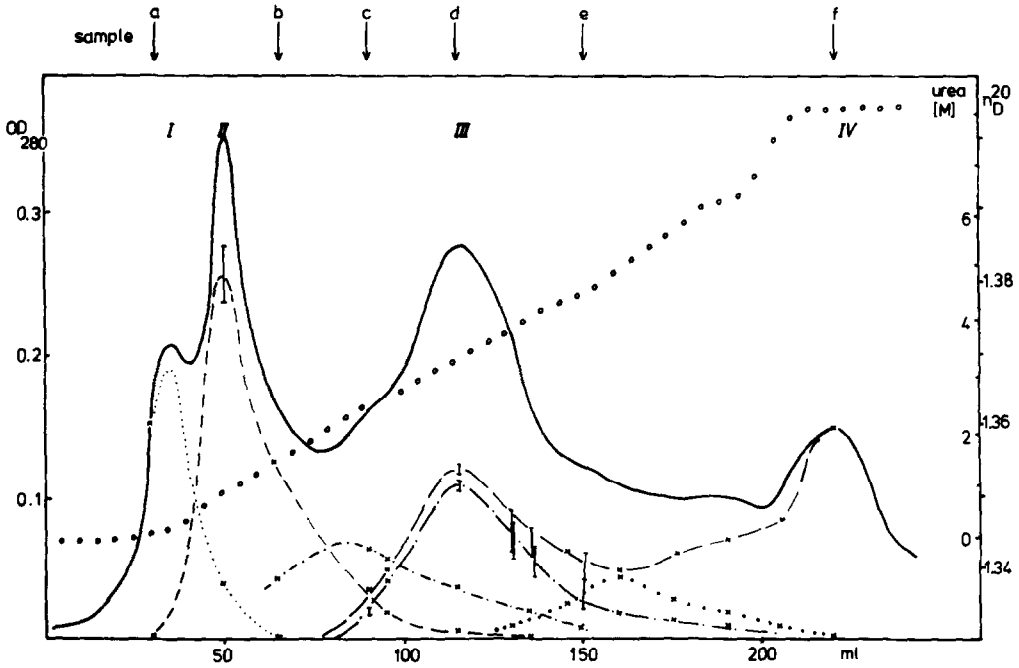

Fig. 2. Elution profile for 100 mg whole histone, chromatographed on 25 ml ARE-agarose, prewashed with 6 M urea

A gradient maker was filled with
100 ml 1 M NaCl, 0.05 M EDTA, 0.02 % NaN_3 (pH 4.5) and
100 ml 1 M NaCl, 0.05 M EDTA, 0.02 % NaN_3,8 M urea (pH 4.5).
Fractionation occured at a flow rate of 10 ml/h and 4° C; after the passage of 200 ml the elution was continued with the final buffer.

Contributions of individual histones to total protein (———) were estimated by gel electrophoresis (fig. 3) and assigned on the basis of their mobilities (19, 20) and their microheterogeneity (KAP: 3-4, ARE: 3, GRK: 2 subfractions; cf ref. (19): (·····), KAP; (- - - - -), KAS; (- · - · - ·), LAK; (— — —), ARE; (— · —), GRK; (• • • • •), fraction "?"; (oooo), refractive index (n_D^{20}) as measure of the urea concentration.

KAS (20, 25, 26) in relation to their helix-content and -stability (26). Consequently, on the basis of its negligible shift in presence of Triton,peak II (i. e. traces b_1/b_2)was assigned to pure histone KAS. Histone LAK appears in front of peak III (trace c_2) only partly overlapping with KAS (fig. 2). The fact that the lysine rich histones KAS and LAK are retarded by ARE-agarose indicates the interaction at least of LAK with matrix bound histone. It is probably this cross

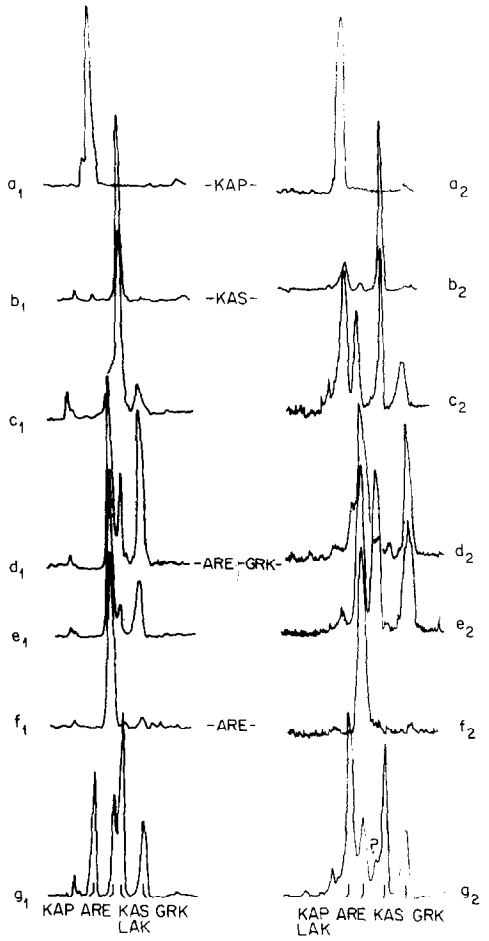

Fig. 3. Gel electrophoretic analysis of histone fraction from ARE-agarose

Samples were taken from the separation of fig. 2 (arrows). Gels were prepared according to ref. (19)(traces a_1 to g_1) and to ref. (20) (traces a_2 to g_2) and stained with comassie brillant blue. Densitometric traces were recorded at 580 nm using a Gilford spectrophotometer with a model 2410 linear transport attachment. Traces g_1 and g_2 represent the composition of whole histone.

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interaction which weakenes the KAS-LAK contact and prevents a complete coelution.

Peak III (fig. 2) comprises histones ARE and GRK which cochromatograph in a constant ratio close to 1 : 1. It is followed by an intermediate region, dominated by ARE and a fraction superimposed by ARE on conventional gels but well seperated in presence of Triton

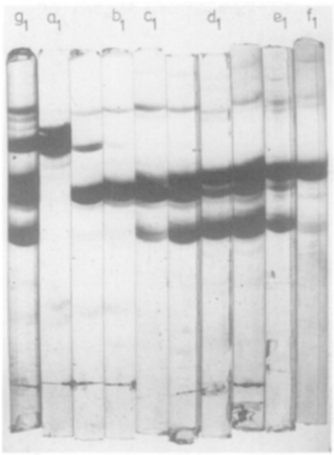

Fig. 4. Photographic representation of gels a_1 to g_1 and some intermediate fractions

(traces e_1 and e_2 resp.). The possibility exists that we have to deal with the monosulfhydryl variant of ARE previously suggested (27) since a cysteine content of this fraction is indicated by its coupling to pCMB-sepharose (fig. 1 b, minor band). Pure histone ARE finally constitutes the terminal peak (IV) eluted by 8 M urea.

The apparent predominance of histone ARE found in fig. 2 is due to its incomplete removal during a prewash with 6 M urea. It is only under these conditions that the total GRK occurs in peak III where it coexists with ARE. If the natural histone mixture applied to the column is not supplemented by additional ARE (prewash with 8 M urea), strongly GRK enriched fractions are found between peaks III (ARE-GRK) and IV (ARE); the latter are then both reduced in size.

To gain some insight into the capacity of ARE-agarose, 25 ml were charged with 500 mg of whole histone. The protein excluded in 1 M NaCl, 0.05 M EDTA, (46 % of the total) was found to contain the major part of the KAS-LAK fraction besides histone KAP which appeared quantitatively. 27 % of fraction GRK could be recovered from this band but virtually none of histone ARE.

CONCLUSIONS

ARE agarose has a capacity for histones, which is only paralleled

by ion exchange resins (> 4 <20 mg/ml). Elution under relatively mild conditions by successive unfolding of bound protein occurs in a manner unterstandable on the basis of mutual histone affinities. These findings are highly indicative for a stoichiometric complex of the arginine rich histones which appears as peak III at 3-4 M urea. The fact that ARE is desorbed last demonstrates that its self aggregation is favored over its cross interaction with GRK. It is concluded therefore that in $(ARE)_n(GRK)_n$ complexes the ARE-ARE-binding domains are stronger than those between ARE and GRK[+)]. The dissociation association equilibrium of the system can therefore be described as follows:

$$(ARE)_n(GRK)_n \rightleftharpoons (ARE)_n + (GRK)_n \rightleftharpoons n(ARE) + n(GRK)$$

Organomercurial agarose appears to be generally applicable for subunit exchange chromatography of sulfhydryl containing oligomers provided that the reaction can be directed towards a single protomer.

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+) While this work was in progress, Mizon et al. published findings on histone affinities towards fraction GRK attached to CNBr activated agarose (28). KAP and LAK/KAS were eluted under non denaturing conditions; remaining histone (GRK and very little ARE) was then released by 0.01 N HCl. A complexation between the arginine rich species could not be observed under the conditions applied nor could the sequence of their elution be established.

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