ALTERATION OF HUMAN INTRINSIC FACTOR DURING AFFINITY CHROMATOGRAPHY PURIFICATION USING CONCENTRATED GUANIDINE

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1. Introduction

Affinity chromatography using vitamin B_{12} (B_{12}) covalently coupled to Sepharose has been used to isolate from solution a number of B_{12}-binding proteins including gastric intrinsic factor (IF) [1,2]. The procedure requires the use of concentrated guanidine–HCl, a known protein denaturant, to dissociate the binding protein from the B_{12}–Sepharose complex.

The B_{12}-binding proteins, like other ligand-binding transport macromolecules, are structurally bifunctional; that is, there are two biologically important reacting sites, one which binds the ligand-substrate and the second which attaches to a receptor site on the cell. When such a binding protein is isolated by affinity chromatography using the ligand-substrate coupled to the solid matrix, the ligand binding site on the macromolecule becomes structurally fixed, whereas the receptor binding site remains exposed and more susceptible to chemical alteration. This report will present evidence that human gastric intrinsic factor purified by elution from B_{12}–Sepharose column by 7.5 M guanidine–HCl, although structurally homogeneous by disc-gel electrophoresis, is nevertheless different than the native IF in gastric juice because there is decreased affinity for the intestinal receptor.

2. Materials and methods

2.1. *Purification of human IF by affinity chromatography*

Human IF was purified by affinity chromatography

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according to the method of Allen et al. [2]. Briefly, normal human gastric juice (NHGJ) obtained by naso-gastric suction after pentogastrin stimulation [3] was applied to an affinity resin composed of vitamin B_{12} covalently coupled to Sepharose beads (Pharmacia Fine Chemicals) via a six carbon (3,3'-diaminodipropylamine) spacer group. After thorough washing of the resin with 0.1 M Tris–acetate, pH 9.2, 0.1 M glycine–NaOH, pH 10, containing 0.1 M glucose and 1.0 M NaCl and 0.1 M potassium phosphate, pH 7.5, IF was eluted by exposure of the resin to 7.5 M guanidine–HCl in 0.1 M potassium phosphate, pH 7.5, for 1 h. A longer period of exposure to guanidine–HCl (18 h) is required to dissociate the R-type B_{12} binder in gastric juice. The fraction containing the IF was then dialyzed against 7.5 M guanidine–HCl for 48 h and then against water, after addition to the sample of a three-fold excess of cyano [57Co]cobalamin ([57Co]B_{12}) (0.01 μCi/μg) (Amersham/Searle Corp.). A fraction of the purified IF was dialyzed against water which contained no B_{12} and this is referred to as unsaturated IF. Disc-gel electrophoresis [4] of the final preparation showed one protein band. Similar electrophoresis of a sample saturated with [57Co]B_{12} and iodinated with 125I using chloramine T [5] demonstrated that the [57Co]B_{12} and 125I peak moved together through the gel.

The B_{12} binding capacity of the purified unsaturated IF was determined by incubating an aliquot of the preparation with a saturating amount of [57Co]B_{12} for 30 min and then precipitating the bound vitamin using $ZnSO_4$ $Ba(OH)_2$ as described previously [6].

The interaction of the purified IF with blocking (type I) and binding (type II) anti-IF antibody was

determined by the method of Rothenberg et al. [7] using the serum from a patient with pernicious anemia as the source of both types of antibody. The same radioassay procedure was used to measure the amount of B_{12} specifically bound to IF in NHGJ.

The interaction of the purified IF with the receptor protein prepared from the guinea-pig ileal mucosa was determined by the method of Cotter and Rothenberg [8].

3. Results

There was a distinct difference in the interaction of the purified IF with excess blocking and binding anti-IF antibody as shown by the data in table 1. Whereas approximately 84% of the B_{12} binding was blocked by preincubating the IF with the antiserum, only 42% (30 min incubation at 22°C) to 54% (18 h incubation at 4°C) of IF-bound $[^{57}Co]B_{12}$ precipitated with the binding antibody. The ratio of binding to blocking antibody reaction was approximately 0.5 for the 30 min incubation. Similar reactions with this antiserum and native IF gave a binding to blocking ratio of 1.13. It is evident from these results that the moiety on the IF molecule which reacts with the binding antibody and which is distant from the B_{12} binding site is altered by the purification procedure. The fact that immunoreactivity with the binding antibody increases 29% over 18 h as compared to only a 3% increase with the blocking antibody reaction, indicates a decreased affinity for the binding antibody

Table 2
Binding of native IF and purified IF by receptor protein from guinea-pig ileum

Substrate	Binding to receptor	
	%	pg[a]
$[^{57}Co]B_{12}$ (65 pg) native IF[b]	67.1	43.6
$[^{57}Co]B_{12}$ (62.5 pg) purified IF	39.5	24.7

[a] Indicates pg of IF bound $[^{57}Co]B_{12}$ — each value is the mean of duplicate reactions
[b] Amount of $[^{57}Co]B_{12}$ bound to IF in NHGJ was determined by radioimmunoassay [7]

site, and is further evidence for a structural alteration of this moiety of the IF molecule. Normally, the binding of native IF by excess antiserum reaches 90% or more of maximum in 30 min.

The binding of the purified IF saturated with $[^{57}Co]B_{12}$ to a preparation of guinea-pig ileum receptor protein was compared to the binding of the same amount of IF-$[^{57}Co]B_{12}$ in NHGJ. Table 2 shows that the receptor preparation bound 67.1%, or 43.6 pg of the $[^{57}Co]B_{12}$ bound to native IF in NHGJ, whereas the receptor bound only 39.5%, or 26.6 pg of $[^{57}Co]B_{12}$ bound to the purified IF.

The relative affinity of the purified IF and native IF in gastric juice for the ileal receptor protein was also determined by measuring the competitive effect of each on the receptor binding of native IF-$[^{57}Co]B_{12}$ The graph in fig.1 shows that the purified IF is much less competitive than equimolar amounts of native IF

Table 1
Reaction of the purified IF and native IF with blocking and binding anti-IF antibody

Reaction	% Blocking[a]	% Binding[b]	Binding / Blocking
Purified IF			
30 min incubation 22°C	82	42	0.51
18 h incubation 4°C	84	54	0.64
Native IF in NHGJ[c]			
30 min incubation 22°C	68	77	1.13

[a] % Total $[^{57}Co]B_{12}$ binding which is blocked by preincubation with the antiserum
[b] % Total bound $[^{57}C]B_{12}$ which is precipitated after incubation with the antiserum
[c] With this sample of NHGJ, the % blocking and binding is reduced because approximately 30% of the $[^{57}Co]B_{12}$ is bound by non-IF binder(s)
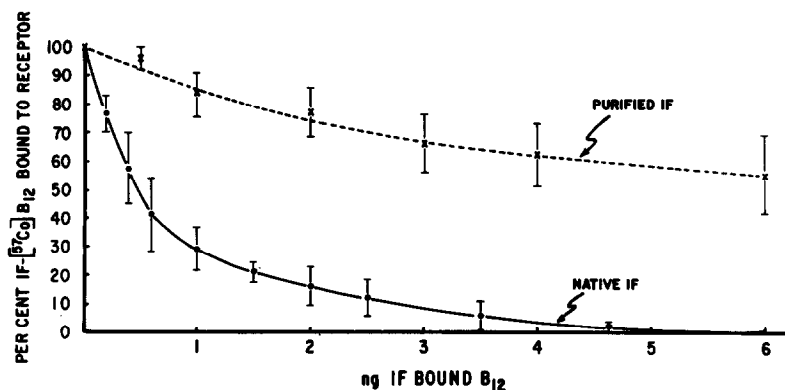
Fig.1. The competitive effect of equimolar concentrations of purified IF-B_{12} (mean ± SD of triplicate determinations) and native IF-B_{12} (mean ± SD of seven different samples of gastric juice) on the binding of native IF-[^{57}Co]B_{12} (100 pg [^{57}Co]B_{12}) by partially purified intestinal IF receptor protein from the guinea-pig ileum. The B_{12} binding capacity of the purified IF was determined by titrating the binding of [^{57}Co]B_{12}. The B_{12} binding capacity of IF in gastric juice was determined by the amount of [^{57}Co]B_{12} specifically precipitated by anti-IF antibody [7]. Each preparation was then saturated with excess unlabeled B_{12} and dialyzed overnight to remove the unbound vitamin. The ordinate was normalized such that the amount of IF-[^{57}Co]B_{12} bound to receptor in the absence of native IF or purified IF was 100%.

(both saturated with unlabeled B_{12}) on the binding of native IF-[^{57}Co]B_{12} to the receptor.

4. Discussion

These data indicate that during the course of affinity purification of human IF using 7.5 M guanidine—HCl, a structural alteration has occurred on that moiety of the molecule which interacts with the binding antibody and the intestinal IF receptor protein with only slight alteration on that part of the molecule which binds to vitamin B_{12} and blocking antibody.

It would seem likely that while the IF is bound to the immobilized B_{12}—Sepharose, the B_{12} binding site (which is also in close proximity to the site which binds to blocking antibody) is protected from the denaturing effects of the guanidine, whereas the more exposed moiety of the IF molecule, which is the reactive site for binding to the intestinal receptor protein (or binding type anti-IF antibody), is unprotected by ligand attachment and is structurally altered by the guanidine.

It is evident that when a protein has two functional moieties, such as a substrate binding site and a receptor binding site, purification by affinity chromatography using strong denaturant chemicals can selec-

tively alter the unassociated (or free) binding site. This may not be appreciated if the structural integrity of the molecule is determined only by its capacity to bind to the ligand to which it was attached during the purification procedure. Studies, therefore, to evaluate the biologic function of the free binding site must be interpreted cautiously since the protein may not be truly representative of the function of the native molecule.

Acknowledgment

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