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A histidine binding protein of *Escherichia coli*: a component of cystine binding protein of *Escherichia coli*

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Summary. Commercially obtained cystine binding protein (CBP), an osmotic shock protein of *Escherichia coli*, was studied in an effort to determine its binding characteristics. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) analysis of commercially obtained CBP showed three protein bands. N-terminal amino acid microsequencing and subsequent computer search revealed that the sequence of one of these proteins (25-kDa) was nearly identical to histidine binding protein (HisJ) of *Salmonella typhimurium*. Purification of CBP by HPLC yielded four protein peaks, of which one bound histidine exclusively. Binding was maximal at pH 5.0 to 6.0, at 4°C, did not require calcium or magnesium ions and was not inhibited by reduction of CBP disulfide bonds. Amino acids other than histidine or cystine did not bind to CBP. These data show that commercially available CBP is not a homogenous protein; it contains a histidine as well as a cystine binding component.

Keywords: Amino acids – Histidine – Cystine – Amino acid binding proteins

Introduction

Commercial CBP is released from the periplasmic compartment of *Escherichia coli* (*E. coli.*) by osmotic shock: Berger (1972). One of its known functions is to facilitate entry of cystine into the cell. Many gram-negative bacterial periplasmic binding proteins are known to promote transport of small molecules: Adams (1989). In particular, it has been demonstrated in *Salmonella typhimurium* (*S. typhimurium*) that histidine transport is a two step process, i) the binding of histidine to HisJ in the periplasmic space and ii) the interaction of the ligand

bound protein with transport proteins in the plasma membrane resulting in delivery of histidine to the cytosol: Higgins (1981). Although CBP has not been fully characterized, it is widely used clinically to quantitate cystine levels in human tissues: Oshima (1974). The purity and biochemical properties of CBP have not been critically evaluated. Therefore, we have further characterized CBP and its binding properties.

Material and methods

CBP was purchased from Riverside Scientific Enterprises, Seattle, WA. Henceforth the use of the term CBP will refer to this commercially available preparation. ^{14}C-labeled amino acids, cystine, methionine, glutamic acid, glutamine, leucine and lysine were obtained from DuPont-NEN, Wilmington, DE. [^{14}C]histidine and [^{14}C]histamine were from Amersham Corporation, Arlington Heights, IL. Nitrocellulose filters, 22 millimeter diameter and 0.45 micron pore size, were from Schliecher and Schuell, Keene, NH. Ready Solv HP Scintillation Fluid was from Beckman Instruments, Inc., Fullerton, CA. Immoblin-P (PVDF) transfer membranes were purchased from Millipore Corp., Bedford, MA.

Cystine binding assay was performed as described substituting non-radioactive and other radioactive amino acids for cystine when applicable: Oshima (1974). Briefly, CBP was incubated with radioactive amino acid in 0.1 M sodium acetate buffer (pH 5) for five minutes, then placed on a water saturated and washed nitrocellulose filter, suction filtered and washed with 0.6 ml of 0.01 M sodium acetate buffer (pH 5). The filter was dissolved in scintillation fluid and counted in a Beckman LS-250 Scintillation Counter. Proteins were determined by the method of Lowry (1951). All experiments were performed in triplicate and results reported as means $\pm$ S.D.

CBP was subjected to SDS/PAGE with and without β-mercaptoethanol by the method of Laemmli (1970). Protein bands were transferred to PVDF membranes by electroblotting and used for N-terminal microsequencing (Harvard MicroChemistry Facility, Cambridge, MA). The N-terminal amino acid sequences were compared with those of known proteins of the PIR data base version 25 in search of similar sequences: Lipman (1985).

CBP (approximately 1 mg per run) was chromatographed by HPLC (Peptide Technologies Corporation, Washington, D.C.) on a reverse phase column (C18 DeltaPak 300A pore, 0.39 $\times$ 30 cm, 15–20 μm packing, Waters, Milford, MA) Proteins were eluted with a multistep curved complex gradient changing from 0% to 35% acetonitrile over 60 min. (solvent A, 0.1% trifluoroacetic acid (TFA) and solution B, 80% acetonitrile in 0.1% TFA) at 0.8 ml/min with detection at 280 nm optical density. The protein content of each separated peak was determined after lyophylization and redissolving a suitable aliquot in 0.5 N NaOH. Using the molecular weight of each protein obtained by gel electrophoresis, the mole content was calculated and 50 pmol aliquots were lyophylized, redissolved in 0.1 M acetate buffer pH 5 with or without 0.4mg/ml bovine albumin and tested for histidine and cystine binding as described above.

Results

CBP of *E. coli* resolved into three bands on SDS/PAGE (10%, pH 8.8) with or without 5 mM β-mercaptoethanol (Fig. 1). CBP was mixed with albumin by the supplier for stability. The higher molecular weight band co-migrated with albumin standard. Thus, the two other bands with apparent molecular weights of 25-kDa (CBP-2) and 23-kDa (CBP-3) were the proteins of interest. These two proteins were transferred to PVDF membrane by electroblotting, stained with Ponceau Red and analyzed for N-terminal sequences. Comparison of the N-terminal sequence data with other known protein sequences in the PIR data

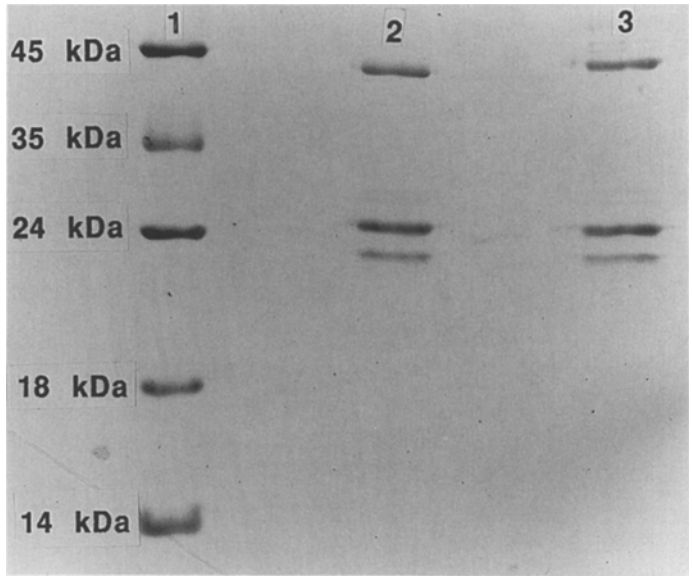

Fig. 1. SDS/PAGE separation of CBP of *E. coli.* CBP and CBP treated with 5 mM β-mercaptoethanol for one hour were subjected to SDS/PAGE (10% gel, pH 8.8). *1* Low molecular weight standards; *2* CBP; Lane *3* CBP treated with β-mercaptoethanol

base revealed the existence of a remarkable similarity (nearly 100%) between *E. coli* CBP-2 and HisJ of *S. typhimurium* (Table 1). Of 25 amino acids sequenced and compared only one conservative substitution was noted. The N-terminal sequence of CBP-3 showed no significant similarity to any known protein in the PIR data base (data not shown).

CBP was then incubated with increasing amounts of [^{14}C]histidine to determine if it bound histidine as well as cystine. Using an estimated molecular weight of 25-kDa for CBP, Fig. 2 shows that CBP binds histidine and that saturation is reached at a molar ratio of 2:1 (histidine 100 pmol: CBP 50 pmol). Scatchard analysis of data in Fig. 2 showed that 3 pmol histidine bound to 50 pmol CBP (see inset) and the apparent binding constant, K_d, was 89 nM. No increased binding of histidine to CBP was observed when the concentration of this amino acid was increased to 400 pmol (data not shown).

We then ascertained the specificity of histidine and cystine binding to CBP. Complete saturation was obtained by incubation of 50 pmol CBP with 200 pmol [^{14}C]histidine. Varying amounts of non-radioactive histidine or cystine were then added to the incubation mixtures (Fig. 3). Radioactive histidine was specifically displaced by non-radioactive histidine in a dose dependent manner. A 50% displacement occurred at non-radioactive histidine concentration of 100 pmol and more than 90% displacement by 1 nmol. Non-radioactive cystine did not displace [^{14}C]histidine bound to CBP. In Fig. 4, the specificity of [^{14}C]cystine binding to CBP is shown. Increasing amounts of non-radioactive cystine displaced the radioactive cystine bound to CBP. Fifty percent displacement was observed at 50 pmol non-radioactive cystine. Non-radioactive histidine did not displace [^{14}C]cystine bound to CBP. When a mixture of [^{14}C]histidine and

Table 1. Comparison of the amino acid sequence of 25 N-terminal amino acids of *E. coli* CBP-2 (reduced protein, 25 kDa) with HisJ of *S. typhimurium*

CBP-2 (*E. coli*)																									
1				5					10					15					20						25
A	I	P	Q	N	I	R	I	G	T	D	P	T	Y	A	P	F	E	S	K	N	S	Q	G		E
				*																					
A	I	P	Q	K	I	R	I	G	T	D	P	T	Y	A	P	F	E	S	K	N	S	Q	G		E
23				27					32					37					42						47

HisJ (*S. typhimurium*)
*Conservative substitution
All amino acids are identical except residues 5 of CBP-2 and 27 of HisJ

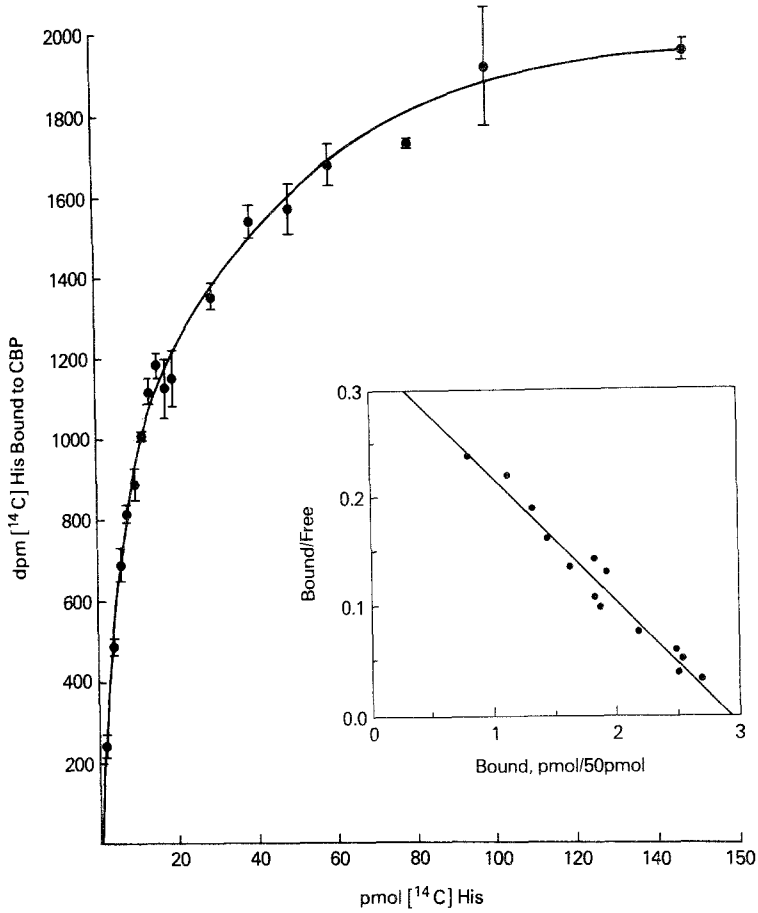

Fig. 2. Binding of [^{14}C]histidine(*His*) to CBP. CBP (50 pmol) was incubated 5 min with increasing amounts of [^{14}C]histidine and processed as outlined in Methods

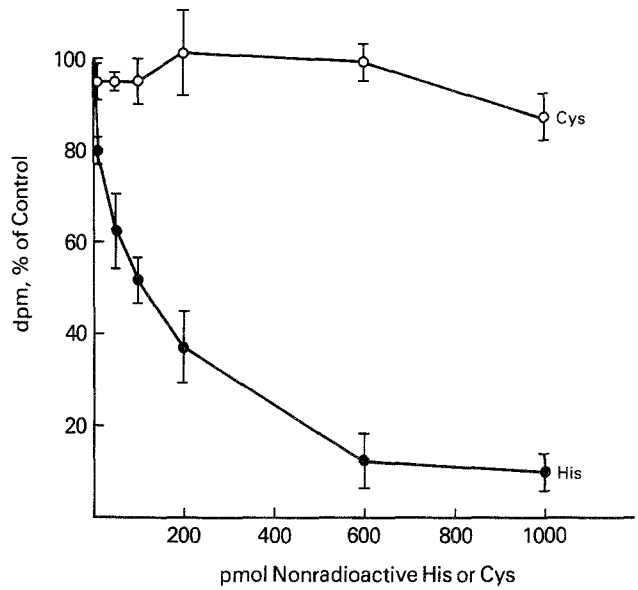

Fig. 3. Displacement of [^{14}C]histidine(*His*) bound to CBP by non-radioactive histidine(*His*) and cystine(*Cys*). CBP (50 pmol) was saturated with 200 pmol [^{14}C]histidine. Non-radioactive His or Cys were then added in the varying amounts indicated and the samples processed as described in Methods. Data points are plotted as % of control binding

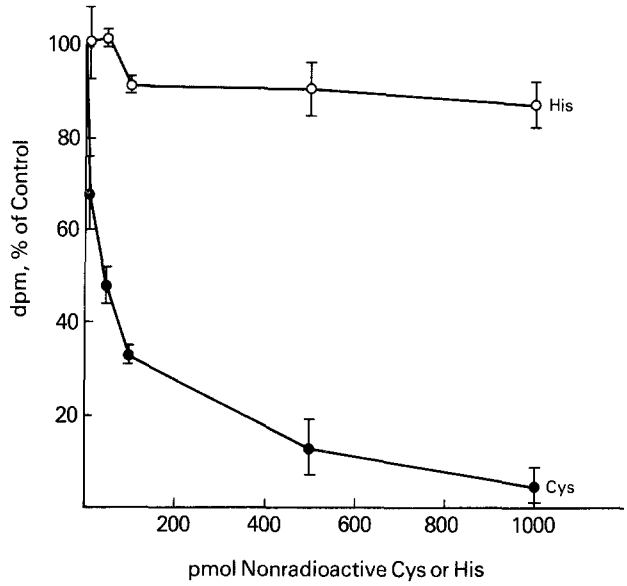

Fig. 4. Displacement of [^{14}C]cystine(*Cys*) bound to CBP by non-radioactive cystine (*Cys*) or histidine (*His*). CBP (50 pmol) was saturated with 200 pmol [^{14}C]cystine. Non-radioactive Cys or His were then added in the varying concentrations indicated and the samples processed as described in methods. Data points are plotted as % of control binding

Table 2. Binding of [^{14}C]histidine and [^{14}C]cystine to CBP

^{14}C-Labeled amino acid	dpm Bound
Histidine	4380 ± 125
Histidine + Cystine	6750 ± 292
Cystine	2278 ± 199
Cystine + Histidine	6347 ± 670

CBP (50 pmol) was saturated with 200 pmol [^{14}C]cystine or [^{14}C]histidine for 5 min, then 200 pmol of the opposite ^{14}C-labeled amino acid was added for 5 min and binding was determined as outlined in Methods

[^{14}C]cystine at a 1:1 molar ratio was incubated with CBP, an additive effect was obtained (Table 2).

Binding of various classes of amino acids to CBP was also tested (Fig. 5). Only cystine and histidine were bound by CBP. Histamine, which is similar in configuration to histidine, lacking only the carboxyl group, bound weakly to CBP.

The temperature dependence of histidine binding to CBP was tested by incubating increasing amounts of [^{14}C]histidine with 50 pmol CBP at 4°C, 23°C, and 37°C. Histidine binding to CBP was optimal at 4°C (Fig. 6). However,

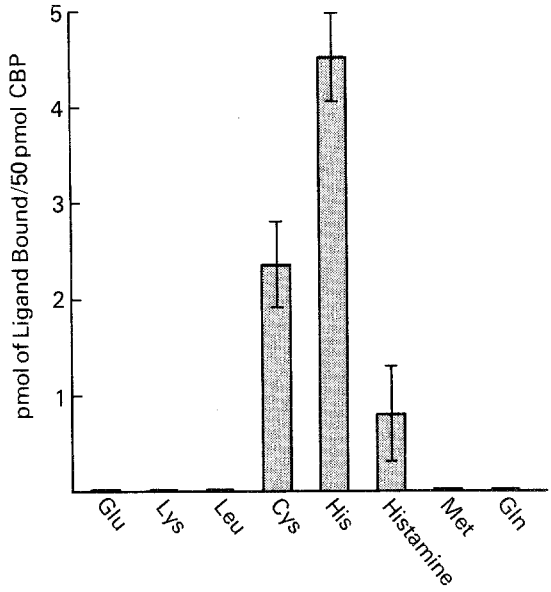

Fig. 5. Binding by CBP with various classes of amino acids. CBP (50 pmol) was incubated 5 min with 200 pmol of each ^{14}C-labeled amino acid indicated and samples processed as outlined in Methods

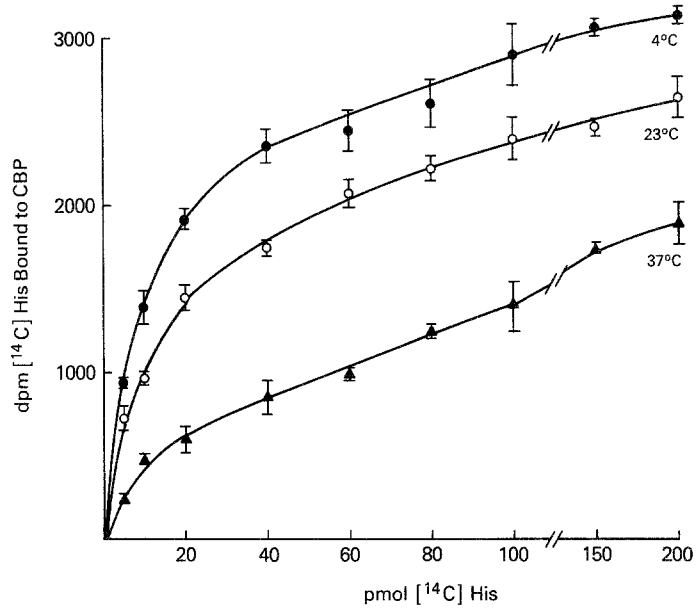

Fig. 6. Temperature effect on [^{14}C]histidine(*His*) binding to CBP. CBP (50 pmol) was incubated with varying amounts of [^{14}C]his at the three temperatures indicated and processed as outlined in Methods

since the cystine binding assay is generally performed at room temperature, all other experiments reported here were carried out at room temperature.

[^{14}C]histidine binding to CBP was tested over a range of pH values and in two different buffers, sodium acetate and sodium citrate-phosphate (Fig. 7).

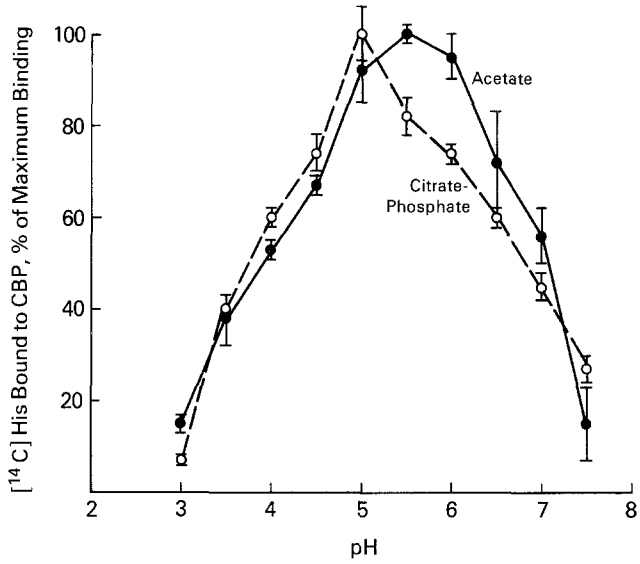

Fig. 7. pH dependence of [^{14}C]histidine(*His*) binding to CBP. CBP (50 pmol) was incubated with 200 pmol [^{14}C]his in sodium acetate or sodium citrate-phosphate buffer at the various pHs indicated and processed as outlined in Methods

Maximum binding was achieved at pH 5.0 in citrate-phosphate buffer and at pH 5.0 to 6.0 in acetate buffer. Since the N-terminal microsequencing analyses were performed on protein bands of CBP resolved by SDS/PAGE (pH 8.8) under reducing conditions, we treated CBP with 5 mM β-mercaptoethanol for one hour in a 50 mM K_3PO_4 buffer (pH 7.0) to reduce any disulfide bonds and then tested for histidine binding. In the final assay, the pH was returned to 5.0 with 0.1 M sodium acetate buffer resulting in a 10 fold dilution of β-mercaptoethanol. Even after such treatment, CBP still bound histidine up to 86% of control values (Table 3). Cystine binding to reduced CBP was also tested but in this case β-mercaptoethanol was removed (to prevent reduction of cystine) by evaporation under nitrogen gas and redissolved in 0.1 M acetate buffer containing 1 mM EDTA to prevent reoxidation of the reduced products. Binding of cystine to reduced CBP was decreased to 79% of control (Table 3). Control experiments showed that EDTA did not interfere with either histidine or cystine binding to CBP (data not shown). [^{14}C]histidine binding to CBP was also assayed in the

Table 3. Comparison of amino acid binding to CBP or reduced CBP

	dpm Bound to		
Amino acid	CBP	Reduced CBP	% of Control
Histidine	3085 ± 200	2661 ± 77	86
Cystine	1732 ± 86	366 ± 18	21

50 pmol CBP were incubated with 200 pmol ^{14}C-labeled amino acid and processed as described in Methods

presence of 1 mM $CaCl_2$ as well as 1 mM $MgCl_2$. Binding was not affected by either of these ions (data not shown).

The proteins in CBP were then separated on a hydrophobic HPLC column (as explained in Methods) and fractions collected. CBP resolved into four well separated protein peaks (Fig. 8). Fractions representing the mid-portion of each peak were assayed for protein content and for histidine and cystine binding. Table 4 shows that CBP and peak I bound histidine. Peaks II, III and IV bound less than 6% of histidine. CBP and Peak III bound cystine. Five μg of each peak and of CBP were then separated on a 15% SDS/PAGE (Fig. 9). Peak I corresponded to CBP-2 (25kDa) of Fig. 1, the protein which had nearly 100%

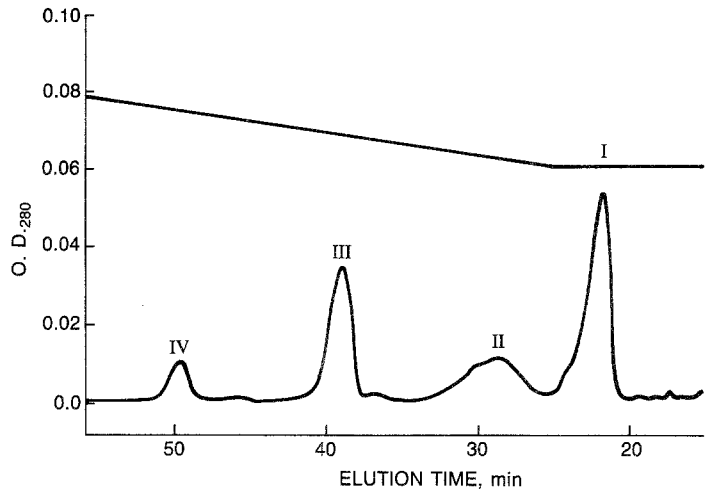

Fig. 8. HPLC elution profile of CBP of *E. coli.* Approximately 1 mg CBP was loaded on a reverse phase HPLC column and proteins were eluted with an acetonitrile gradient changing from 0% to 35% acetonitrile over 60 min (solvent A, 0.1% trifluoroacetic acid (TFA) and solution B, 80% acetonitrile in 0.1% TFA). The flow rate was 0.8 ml/min and proteins were detected at 280 nm optical density

Table 4. Binding of [^{14}C]histidine and [^{14}C]cystine to protein derived by separation of CBP by HPLC

Protein	Histidine cpm Bound	Cystine cpm Bound
CBP	6757 ± 183	5246 ± 392
Peak I	7542 ± 985	146 ± 65
Peak II	408 ± 208	101 ± 50
Peak III	119 ± 91	4134 ± 235
Peak IV	198 ± 205	107 ± 50

CBP was separated by HPLC, eluted with an acetonitrile gradient (see Fig. 8), 1 ml fractions collected and the center of each peak tested in triplicate for histidine binding. Using estimated molecular weights derived from Fig. 1, and protein values determined by Lowry (1951), 50 pmol of each peak were incubated with 200 pmol of radioactive histidine and cystine and processed as outlined in Methods

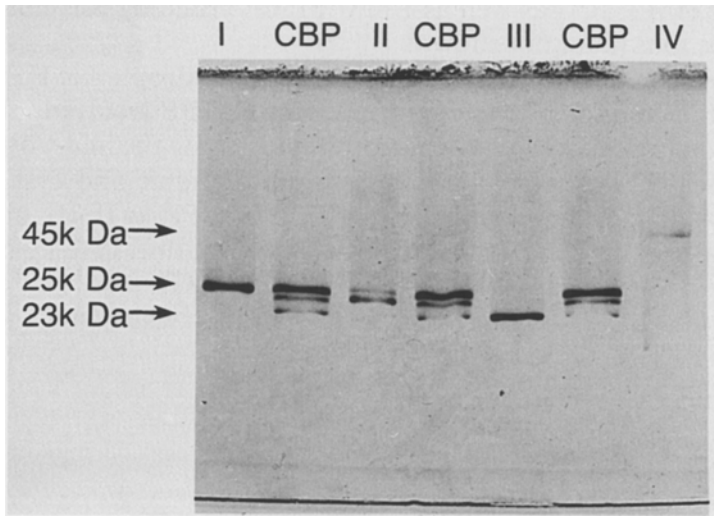

Fig. 9. SDS/PAGE separation of proteins eluted from the HPLC column shown in Fig. 8 and of CBP. Five μg of CBP and of each protein peak eluted from the HPLC column were separated by 15% SDS/PAGE. Peaks *I*, *II*, *III*, and *IV* are shown interspersed with CBP

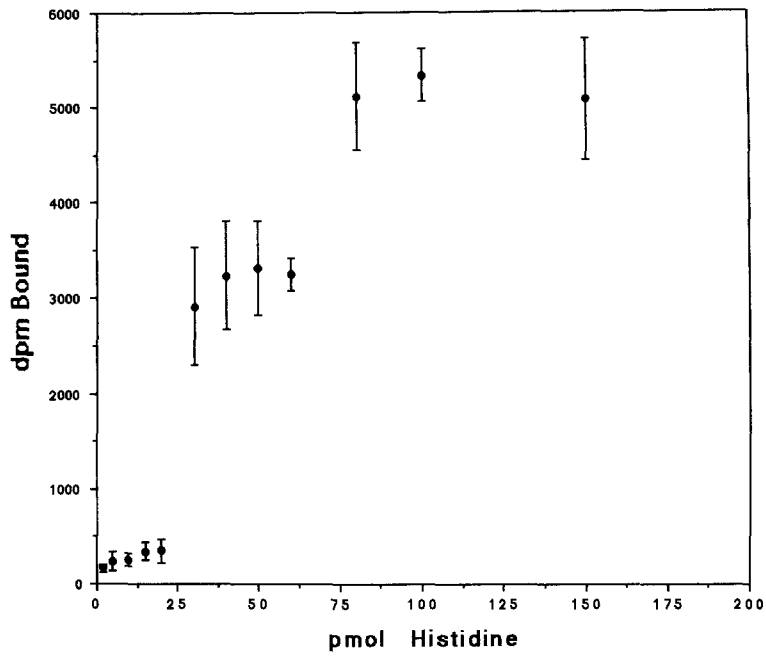

Fig. 10. Binding of [^{14}C]histidine to pure histidine binding protein (Peak I) isolated from CBP. Aliquots of peak I containing 50 pmol of protein were incubated with increasing amounts of [^{14}C]histidine and amino acid binding assayed as outlined in Methods

N-terminal sequence similarity to HisJ of *S. typhimurium*. Peak III corresponded to CBP-3 (23kDa) of Fig. 1, the protein which had no sequence similarity to any known sequence in the PIR data base. Peak II was a mixture of two proteins not resolved by HPLC but resolved into 2 bands by gel electrophoresis. Peak IV runs with the albumin standard and is probably the albumin added by the

supplier for stability. The albumin concentration in this preparation was so low it did not show on the gel when 5 μg CBP was applied. Using 15% SDS/PAGE allowed resolution of an extra band in CBP not seen in the 10% SDS/PAGE of Fig. 1.

Aliquots (50 pmol) of the purified histidine binding protein (Peak I) were then incubated with increasing amounts of [^{14}C]histidine to study its saturation characteristics (Fig. 9). Unlike results seen in Fig. 2, three saturation plateaus were observed, one at 5 to 20 pmol, another at 30 to 60 pmol and a third at 80 to 150 pmol. Histidine binding activity of the purified protein (Peak I) varied with storage time (at -20°C), and binding was only reliably demonstrable on relatively freshly isolated protein. Binding activity was increased 50% and stabilized for histidine binding protein that had been stored frozen for one month by the addition of 0.4 mg/ml bovine albumin. Therefore the saturation curve using freshly obtained purified histidine binding protein was repeated with 0.4 mg/ml bovine albumin in the buffer. Protein binding activity was increased 50% and the three saturation plateaus were again observed (data not shown). Because of the non-hyperbolic nature of these curves, Scatchard analysis for determination of the number of binding sites and the K_d could not be reliably performed using purified histidine binding protein.

Discussion

Our data show that commercially available CBP obtained by osmotic shock of *E. coli* is a combination of binding proteins, one of which binds histidine exclusively. The newly discovered histidine binding of CBP is saturable and specific. Bound radioactive histidine is displaceable by non-radioactive histidine but not by cystine, and bound radioactive cystine is displaceable by non-radioactive cystine but not by histidine. Binding of both radioactive amino acids was additive (Table 2). Final proof that CBP binds histidine independently was demonstrated by its separation on a hydrophobic HPLC column and subsequent testing of these separated proteins for binding activity with the results that only Peak I bound histidine and only Peak III bound cystine. Thus, CBP, as a crude commercially available preparation, has now been shown to bind histidine as well as cystine. Scatchard analysis of data from the saturation curve of crude CBP showed a low K_d indicating a strong binding affinity for histidine. The apparent number of binding sites was also low but this may reflect some denaturation of the protein in the extraction procedures. The fact that the purified *E. coli* histidine binding protein is somewhat unstable supports the need for the stabilizing effect of albumin present in the commercial preparation.

CBP-2 of the SDS/PAGE separation of CBP showed remarkable N-terminal sequence similarity to HisJ of *S. typhimurium*. HisJ has been extensively studied: Higgins (1981). Its function is very similar to a general bacterial periplasmic transport mechanism, which upon binding its ligand undergoes a conformational change enabling it to bind to a complex of plasma membrane transport proteins which then carry the ligand into the cell. We speculate that a similar ligand receptor and conformational change may be operating in the histidine binding protein of *E. coli* to facilitate histidine transport. Support for this concept

is provided by the finding that purified histidine binding protein of *E. coli* did not exhibit a smooth saturation curve as was seen for the crude CBP (Fig. 2) but instead had three plateau areas (Fig. 9), a phenomena which persisted even when binding was inhanced 50% by addition of albumin to the incubation medium. This preliminary finding indicates that a cooperative mechanism may be operative and that a chaperone protein(s) may be needed in vivo.

Histidine binding by CBP was better at 4°C (Fig. 6). At this low temperature weaker binding forces such as van der Waals and hydrogen bonding may be more effective. Histidine binding did not require disulfide bonds but was pH-dependent (optimum at pH 5 to 6) indicating involvement of ionic interactions as well. At pH 5 where the assays were run, histidine would have a net positive charge. Lack of the carboxyl group of histidine, as occurs in histamine, caused a large decrease in binding of this ligand. Therefore, this ionizable group may contribute substantially to the overall binding of histidine.

CBP is routinely used to determine cystine levels in tissues of children affected with the autosomal recessive genetic disease, cystinosis: Oshima (1974). Since CBP binds both cystine and histidine without reciprocal interference, this commercial preparation can continue to be used for cystine analysis. In addition, CBP could be adapted to measure histidine in a manner identical to the CBP assay for cystine and used to study tissues whose levels of histidine need quantitation in a highly sensitive assay.

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