A Single Amino Acid Substitution in a Histidine-Transport Protein Drastically Alters Its Mobility in Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis†

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ABSTRACT: Mutation *hisJ5625* has altered the histidine-binding protein J of *Salmonella typhimurium* such that histidine transport is impaired, even though binding of histidine by the J protein is unimpaired [Kustu, S. G., & Ames, G. F. (1974) *J. Biol. Chem. 249*, 6976–6983]. We have determined by protein analytical methods that the only effect of this mutation has been the substitution of a cysteine residue for an arginine at a site in the interior of the polypeptide chain. This arginine residue is therefore potentially essential for the transport function of the protein. The mutant protein migrates in sodium dodecyl sulfate–polyacrylamide gel electrophoresis more slowly than the wild type protein, as if its molecular weight were greater by as much as 2000. Since this behavior is apparently due to a single amino acid replacement, a molecular weight difference even between two closely related proteins should not be inferred solely on the basis of sodium dodecyl sulfate gel electrophoresis.

The histidine-binding protein J of *Salmonella typhimurium* is a periplasmic protein involved in the transport of histidine (Ames & Lever, 1970). Mutation *hisJ5625* structurally alters the J protein and impairs histidine transport but does not affect the histidine-binding activity of the isolated protein (Kustu & Ames, 1974). Rather, it is believed to affect a proposed interaction in vivo between the J protein and another histidine-transport protein, the P protein (Ames & Spudich, 1976).

The intriguing nature of this mutation prompted more detailed study of the structural alteration in the *hisJ5625* mutant histidine-binding protein. One manifestation of the alteration is that the mutant J protein has a decreased mobility in $NaDodSO_4$[1]–polyacrylamide gel electrophoresis (Kustu & Ames, 1974). Since $NaDodSO_4$ gel electrophoresis has been considered to separate proteins mostly as a function of molecular weight (Shapiro et al., 1967; Weber & Osborn, 1969), it seemed likely that the *hisJ5625* J protein was larger than the wild type protein. Because of the periplasmic nature of the J protein, it was particularly interesting to determine whether the protein was truly larger and whether it possessed an extra sequence at the amino or at the carboxy terminus of the protein. Therefore, we undertook a thorough analysis of the alteration in this mutated J protein.

Materials and Methods

Materials. All chemicals were of the highest grade available from the respective supplier: ammonium sulfate ("Ultrapure") and guanidine hydrochloride (Gdn·HCl) ("Ultrapure") from Schwarz-Mann; iodoacetamide and iodoacetic acid from Sigma; radioactive iodoacetic acid ([2-^{3}H]) from Amersham/Searle; cyanogen bromide and mercaptoethanol from Eastman; dansyl chloride from Pierce; carboxymethylcellulose, hydroxylapatite, and Bio-Gel P-100 from Bio-Rad; Cheng-Chin polyamide sheets from Gallard-Schlesinger or Pierce; diethylaminoethylcellulose (DEAE-cellulose) from Whatman (DE-32), formic acid and (2-bromoethyl)trimethylammonium bromide (BETAB) from Aldrich; phenanthrenequinone from Matheson Coleman and Bell; thin-layer cellulose polygram Cel 300 from Brinkman; trypsin (TPCK-treated) from Worthington. $NaDodSO_4$–polyacrylamide gel electrophoresis reagents have been specified previously (Ames, 1974). All other chemicals were reagent grade.

Protein Purification. The histidine-binding protein J and the mutant J protein were purified by the same procedure, except that the mutant protein was maintained in 0.1% (v/v) mercaptoethanol throughout. The source of the J protein was *S. typhimurium* strain TA1014 (Kustu & Ames, 1974); the mutant J* protein was isolated from strain TA300 (Kustu & Ames, 1974). Cells were grown in minimal medium (Vogel & Bonner, 1956) supplemented with 0.4% (w/v) glucose to stationary phase of growth (2.5×10^9 cells/mL). The cells were harvested by centrifugation, suspended in 10 mM potassium phosphate, pH 7.6, ruptured by three passages through a Manton-Gaulin press, and centrifuged at 27000*g* for 90 min at 0–4 °C. All subsequent procedures were performed at 0–4 °C. Ammonium sulfate was added to the supernatant cell extract to a concentration 60% of saturation (Di Jeso, 1968). The supernatant was brought to 100% saturation with ammonium sulfate, and the precipitate was dialyzed against 5 mM Tris-HCl, pH 8.3. This sample was applied to a 4.2 × 24 cm DEAE-cellulose column in the same buffer and eluted with a gradient of NaCl from 0 to 50 mM in 5 mM Tris-HCl, pH 8.3 (total gradient volume 4 L). The pooled fractions containing J protein were concentrated with a PM10 Diaflo membrane (Amicon Corp.) and dialyzed against 10 mM potassium acetate, pH 5.0. This sample was applied to a 3.4 × 24 cm carboxymethylcellulose column and eluted with a gradient of NaCl from 0 to 0.2 M in 10 mM potassium acetate, pH 5.0 (total gradient volume 3 L). The pooled fractions containing J protein were concentrated with a PM10 Diaflo membrane and dialyzed against 1 mM potassium phosphate, pH 6.8. The mutant protein was further purified on a hydroxylapatite column in 1 mM potassium phosphate,

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; BETAB, (2-bromoethyl)trimethylammonium bromide.

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pH 7.0, with a gradient of 1 to 100 mM potassium phosphate.

Reduction and Alkylation. The lyophilized protein at 0.5 or 4 mg/mL was reduced in 0.4 M Tris-HCl, pH 8.6, 0.2% NaEDTA, and 5 M Gdn·HCl with 0.14 M mercaptoethanol; after 4–8 h at 25 °C, it was alkylated with iodoacetamide by the method of Crestfield et al. (1963). Labeling of the cysteine residues was accomplished by replacing iodoacetamide with radioactive [2-^{3}H]iodoacetic acid. One milligram of protein was reduced in 0.89 mL of the reducing solution in the dark, after which 20 μL of 0.1 M (1.02×10^8 cpm) iodoacetic acid was added; after 10 min 66 μL of 50% (w/v) iodoacetate was added to complete the alkylation and, subsequent to 15 min further incubation, 10 μL of mercaptoethanol was added to quench the unreacted iodoacetate. The pH was maintained between 8.2 and 8.6 during all alkylation reactions.

Sodium Dodecyl Suflate (NaDodSO$_4$)–Polyacrylamide Gel Electrophoresis. Either the discontinuous Tris–glycine buffer system (Ames, 1974; Laemmli, 1970) or the continuous phosphate buffer system (Shapiro et al., 1967) was utilized for NaDodSO$_4$–polyacrylamide slab gel electrophoresis. To separate polypeptides in the 3000–10 000 molecular weight range, we employed NaDodSO$_4$ gels with a gradient in acrylamide concentration (from 10 to 22.5%). The discontinuous Tris–glycine buffer system was used with a stacking gel of 5% acrylamide.

Amino Acid Analysis. The protein or peptide (5–20 nmol) in water or volatile buffer was pipetted into a nitric acid washed tube (6 × 150 mm) which had been heated at 400–500 °C overnight. After the sample was lyophilized, 10 drops of 6 N HCl (analytical reagent grade) was added and the tube was sealed in a flame under vacuum. After being heated at 110 °C for 24–72 h, the tube was stored at 4 °C or opened immediately. Once opened, the samples were dried under vacuum at room temperature over NaOH and stored dry at −20 °C. A few hours before being run on an analyzer, the dry hydrolysate was dissolved in loading buffer, 67 mM sodium citrate, pH 2.2. Various Beckman analyzers of the single-column or double-column type were employed. Homoserine was analyzed according to the method of Ambler & Brown (1967).

CNBr Cleavage. At a concentration of 13 mg/mL, protein alkylated with iodoacetamide was reacted with 100 mM CNBr in 70% formic acid for 24 h at room temperature in the dark in a total volume of 2 mL. The solution was diluted with a 10-fold volume of water and lyophilized. This step was repeated 2 more times. The lyophilized sample was then dissolved in NaDodSO$_4$ gel sample buffer (Ames, 1974) for analysis on NaDodSO$_4$ slab gel electrophoresis. For amino acid analysis 25 mg of the CNBr fragments was purified on a Bio-Gel P-100 column. The lyophilized fragments were dissolved in 4 mL of 10% (v/v) acetic acid at 50 °C, applied to a 2.5 × 87 cm column equilibrated in 10% acetic acid, and eluted with 10% acetic acid at 25 °C. The emergence of polypeptide was monitored by absorbance at 280 nm.

The CNBr fragment deriving from the amino-terminal portion of the protein was identified by CNBr cleavage subsequent to dansylation. Protein (0.15 mg) alkylated with iodoacetamide was reacted with dansyl chloride (Weiner et al., 1972) and precipitated with 10% (w/v) trichloroacetic acid. After being washed with 1 mL of 1 N HCl, 1 mL of acetone, and 1 mL of 1 N HCl and finally dried under vacuum, the precipitate was dissolved in 70% formic acid and reacted with 300 mM CNBr. After 24 h the mixture was lyophilized 3 times. Since the dansylated CNBr fragments did not dissolve readily in water, 0.1% NaDodSO$_4$–10 mM NH$_4$HCO$_3$ was used as the lyophilization buffer. The residue was dissolved in NaDodSO$_4$ sample buffer and subjected to NaDodSO$_4$–polyacrylamide gradient slab gel electrophoresis. The dansyl peptide bands on the gel were located under UV lamp, cut out of the gel, and chopped into pieces 1- or 2-mm square. Gel pieces from each band were covered with 0.1% NaDodSO$_4$–0.05 M NaHCO$_3$, pH 10, and shaken overnight at 37 °C. The supernatant was lyophilized and dissolved in 100 μL of water. To 50 μL was added 50 μg of BSA and 500 μL of ice-cold acetone in an acid hydrolysis tube. After 60 min on ice the fluffy precipitate was centrifuged at 1000*g* for 10 min and dried under vacuum at 25 °C. After the addition of 100 μL of 6 N HCl, the tubes were sealed under vacuum and heated at 105 °C for 6 h. The tubes were opened, and the contents were dried under vacuum at 25 °C. The samples were then extracted twice with 50 μL of water-saturated ethyl acetate. The material extracted into ethyl acetate was dried under vacuum and dissolved in 3 μL of 50% (v/v) pyridine. Chromatography on polyamide plates (Weiner et al., 1972) was performed to identify any dansyl amino acids present.

Tryptic Digestion. J protein, 1.5 mg in 1 mL of 0.01 N HCl, was heated in boiling water for 30 min to denature it. NH$_4$HCO$_3$ was added to a concentration of 0.1 M, and the pH was adjusted to 8–8.5 with NH$_3$. A 20-μL aliquot of TPCK-trypsin (1 mg/mL) in 0.001 N HCl was added, and the resulting sample was incubated at 37 °C for 6 h. The same amount of trypsin was added again, and incubation was continued for another 18 h. After being lyophilized 3 times, the dry residue was dissolved in 0.15 mL of water and 2 μL of 15 M ammonia and stored frozen at −20 °C.

The digests were subjected to two-dimensional separation on thin-layer cellulose by a procedure suggested by T. Leighton, which is similar to that of Chen (1976). A 20-μL sample was electrophoresed at pH 3.5 in 10% (v/v) acetic acid/1–2% pyridine at 400 V for 105 min, with the thin-layer cellulose immersed in Varsol coolant (Exxon Co., Houston, TX). The second dimension of separation was by chromatography with pyridine–butanol–acetic acid–water (50:75:15:60 v/v).

The dried 20 × 20 cm cellulose plate was sprayed with 50 mL of 0.2% (w/v) ninhydrin in acetone, heated 2–5 min in a drying oven, and stored in the dark. Alternatively, the plates were stained specifically for arginine with 25–50 mL of phenanthrenequinone reagent (Easley et al., 1969).

The peptide altered by the mutation was purified from tryptic digests of either mutant or wild type protein by chromatography on Sephadex G-50 (1.5 × 85 cm) in 0.05 M ammonium bicarbonate. It was identified by thin-layer cellulose electrophoresis of fractions from the Sephadex G-50 column. The peptide of interest remained at the origin of electrophoresis and contained arginine (wild type) or cysteine (mutant). The Sephadex G-50 fractions containing the mutant peptide were concentrated by lyophilization. The corresponding wild type peptide required further purification, which was achieved by thin-layer cellulose electrophoresis and elution with 6 N HCl of material remaining at the origin.

Amino-Terminal Analyses. The dansyl–Edman procedure of Weiner et al. (1972) was employed. Identification of a particular dansyl amino acid was confirmed by cochromatography with the respective authentic dansyl amino acid purchased as a standard (Mann Research Laboratories).

Results

Mobility on NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. The structural alteration caused by mutation *hisJ5625* results in a pronounced difference in the mobility

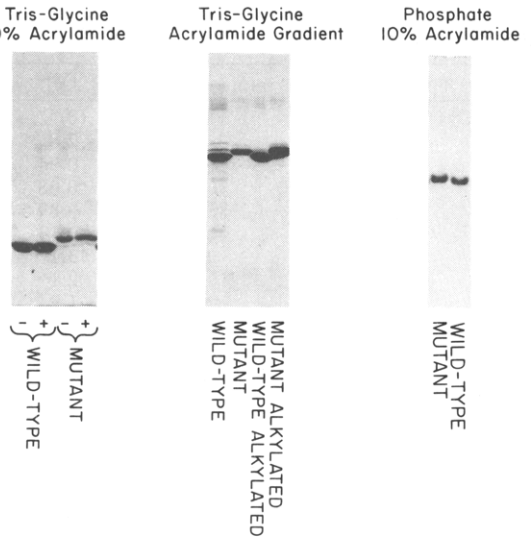

FIGURE 1: Mobility of mutant and wild type J proteins in $NaDodSO_4$–polyacrylamide gel electrophoresis. These are relevant portions of acrylamide slab gels. (Left) A gel utilizing the Tris–glycine buffer system and 10% acrylamide: (+) β-mercaptoethanol added to the sample buffer; (–) no β-mercaptoethanol. (Center) A gel utilizing Tris–glycine buffer and having a gradient in acrylamide from 10 to 22.5%. The proteins were alkylated by reaction with iodoacetamide. (Right) A gel utilizing phosphate buffer and 10% acrylamide in the resolving gel.

of the mutant J* protein on $NaDodSO_4$–polyacrylamide gel electrophoresis (Kustu & Ames, 1974) (Figure 1). The molecular weight of the wild type protein is 25 000 (Lever, 1972); if due to a molecular weight change, the slower migration of the mutant protein on a Tris–glycine-buffered gel corresponds to an increase of 1000–2000 daltons. On a phosphate-buffered gel the respective mobilities of the mutant and wild type proteins correspond to a difference of 500 daltons. A neutral substituent on the sulfhydryl groups produced by alkylation with iodoacetamide does not affect the migration of either protein. Since the proteins were denatured for tryptic digestion by boiling for 30 min at pH 2, it is pertinent that the difference in $NaDodSO_4$ gel mobility survives this treatment.

Preliminary investigations indicated that the mutant protein did not owe its altered mobility to either (a) phosphorylation or (b) glycosylation. (a) Digestion of either wild type or mutant protein with alkaline phosphatase did not change their electrophoretic mobility; no phosphate residue was detected on the wild type protein as analyzed by two-dimensional gel electrophoresis of ^{32}P-labeled cell cultures (G. F.-L. Ames, unpublished experiments). (b) No carbohydrate was found on either protein, assayable either by a periodic acid–Schiff staining procedure performed on acrylamide gels (G. F.-L. Ames, unpublished experiments; Kapitany & Zebrowski, 1973) or by a phenol–sulfuric acid assay (Dubois et al., 1966; Noel, 1977).

Homogeneity of Analyzed Proteins. As indicated in Figure 1, the J and mutant J* proteins used in this study are quite homogeneous as analyzed by $NaDodSO_4$ gel electrophoresis. Less than 1% of the weight of the assayable protein could be attributed to proteins other than J or J*, as determined by inspection of heavily overloaded $NaDodSO_4$ gel electrophoretograms (up to 20 μg of protein per well).

Amino Acid Composition. The amino acid compositions of the two proteins are presented in Table I. There is one obvious difference: the mutant protein contains three cysteine residues, whereas the wild type has only two. If the number of residues is computed by assuming a molecular weight of 25 000 for each protein, then the extra cysteine in the mutant appears to be the result of a substitution of cysteine for an arginine residue in the wild type protein. There is the hint of an extra valine and isoleucine in the mutant also. Other analyses (data not shown) suggest an extra alanine as well. None of these latter differences, however, are as dramatic or as consistently shown as the loss of one arginine and the gain of one cysteine. On the other hand, if the mutant protein is assigned a molecular weight of 26 000 (as indicated by $NaDodSO_4$ gel electrophoresis), it then has several additional amino acids: an aspartate, a serine, a glutamate, an alanine, a glycine, a valine, an isoleucine, a leucine, and a lysine residue. Moreover, the loss of arginine in the mutant is then less obvious: 0.5 residue less than that found in the wild type.

Table I: Amino Acid Composition of the Wild Type and the Mutant J Proteins[a]

amino acid	wild type residues (J)	mutant residues (J*)	J* – J
Cys[b]	2.17	3.11	+0.94
Asp	23.7	23.4	
Thr[c]	12.0	11.9	
Ser[c]	10.9	10.9	
Glu	26.5	26.0	
Pro	8.40	8.25	
Gly	20.6	20.4	
Ala	23.5	23.7	
Val[d]	11.6	12.1	+0.5
Met	3.15	3.13	
Ile[d]	13.8	14.5	+0.7
Leu[d]	17.2	17.4	
Tyr	7.4	7.2	
Phe	11.5	11.7	
Lys	20.9	21.0	
His	1.02	1.10	
Arg	8.50	7.63	–0.87

[a] Aside from the exceptions noted, the values given are averages of 24-, 48-, and 72-h hydrolysates. No analysis for tryptophan was performed. The number of residues is calculated per mole of protein assuming a molecular weight of 25 000 for each protein. [b] Analyzed as (carboxymethyl)cysteine. [c] Extrapolated to 0 h. [d] Extrapolated to 72 h.

These data are not in complete agreement with a previously reported amino acid composition of the wild type J protein from this laboratory (Lever, 1972). The differences stem from an error in the previous analysis of the basic amino acids and perhaps also from the improved purification procedure employed in this study. The data reported here are in reasonable agreement with more recent analyses of these two proteins (Manuck & Ho, 1979).

Amino-Terminal Analysis. By application of the dansyl–Edman procedure of Weiner et al. (1972), the amino termini of the proteins were sequenced. The first four residues are identical for each protein and are H-Ala-Ile-Pro-Glu.

CNBr Cleavage. Figure 2 shows that CNBr cleavage of the protein yields at least four fragments, one of which is incompletely digested (fragment I). The fragments were separated by gradient polyacrylamide gel electrophoresis in a discontinuous $NaDodSO_4$–Tris–glycine buffer system. The four major fragments have approximate molecular weights of 22 000, 15 000, 7000, and 3000. Minor fragments are revealed by the gel (Figure 2) and are due to unexplained cleavage events.

The three CNBr fragments II–IV were purified by chromatography on Bio-Gel P-100 in acetic acid (Figure 3), and an amino acid analysis of each fragment was performed (Table II). Analysis for homoserine revealed that fragment IV (molecular weight ~3000) is apparently the carboxyl-terminal fragment, because it contains no homoserine. Besides peaks

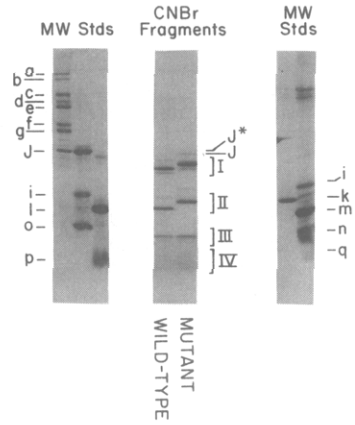

FIGURE 2: CNBr fragments of the wild type and the mutant J proteins. Slab gel with the Tris–glycine buffer system and a gradient in acrylamide from 10 to 22.5%. (Center left) Products of CNBr cleavage of J protein. (Center right) Products of CNBr cleavage of mutant J* protein. (Right and left) Standard proteins: (a) β-galactosidase (130 000), (b) phosphorylase A (94 000), (c) bovine serum albumin (68 000), (d) catalase (60 000), (e) glutamic dehydrogenase (53 000), (f) alcohol dehydrogenase (41 000), (g) glyceraldehyde dehydrogenase (36 000), (h) J protein (25 000), (i) horse heart myoglobin (17 200), (k) egg white lysozyme (14 300), (l) horse heart cytochrome c (12 300), (m) myoglobin CNBr fragment I (8300), (n) myoglobin CNBr fragment II (6400), (o) bovine trypsin inhibitor (6200), (p) glucagon (3500), and (q) myoglobin CNBr fragment III (2500). The molecular weights are according to Weber & Osborn (1969) for (a)–(g), (i), and (k) and Swank & Munkres (1971) for (l)–(q).

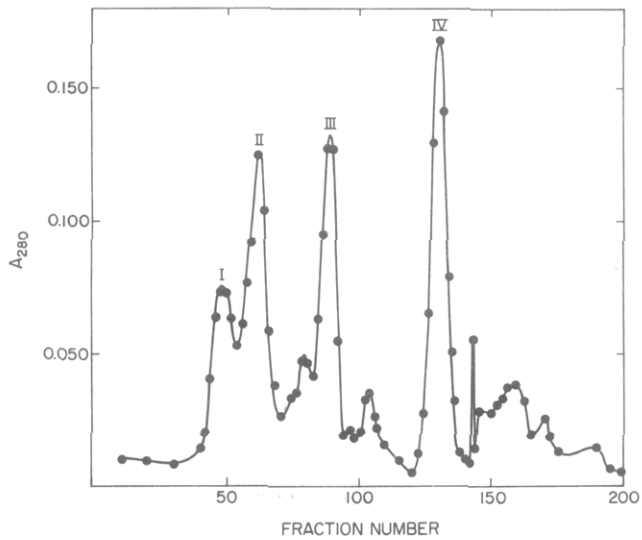

FIGURE 3: Separation of CNBr fragments of J protein on a P-100 column. A 25-mg quantity of J protein reacted with CNBr was applied to a 2.5 × 87 cm column in 10% (v/v) acetic acid. The peaks are labeled according to the convention of Figure 2, as judged by the predominant species on $NaDodSO_4$–polyacrylamide gel electrophoresis.

due to fragments I–IV already discussed, the P-100 column resolves another 280-nm absorbing species (tubes 150–160) which might correspond to a fragment smaller than fragment IV. This material is not visible after $NaDodSO_4$ gel electrophoresis and Coomassie Blue staining, but possibily it is the fourth CNBr fragment predicted by amino acid analysis, which indicated that the protein contains three methionine residues.

In order to identify the CNBr fragment which is derived from the amino terminus of the protein, we reacted the J protein first with dansyl chloride and then subjected it to CNBr treatment. After the fragments were separated by $NaDodSO_4$ gel electrophoresis and digested with 6 N HCl, thin-layer chromatography demonstrated the presence of dansylalanine from fragment III. The other fragments contained only the

Table II: Amino Acid Composition of CNBr Fragments of the Wild Type J Protein[a]

amino acid	II[b]	III[c]	IV[d]
CM-Cys	0.4	0.1	(0)[e]
Asp	15.0	8.0	2.8
Thr	9.7	3.2	0.94
Ser	9.2	3.4	0.08
Glu	18.1	9.0	0.89
Pro	4.4	2.5	0.13
Gly	13.9	10.2	3.1
Ala	14.3	8.0	2.1
Val	11.4	5.5	1.9
Met	0.06	0.03	(0)[e]
Ile	8.4	4.3	0.26
Leu	9.4	4.2	1.05
Tyr	3.5	2.5	2.2
Phe	6.0	4.0	1.8
His	1.05	0.2	0.05
Lys	12.8	6.5	3.0
Arg	4.6	1.6	1.0
homo-Ser	0.89	0.73	(0)[e]

[a] These samples were hydrolyzed for 24 h. [b] Only one determination; residues normalized to 15.0 Asp; molecular weight ~15 000. [c] Average of two determinations; residues normalized to 8.0 Asp; molecular weight ~8000. [d] Only one determination; residues normalized to 1.0 Arg; molecular weight ~2500. [e] None detected.

dansyl derivatives ε-dansyllysine and *O*-dansyltyrosine, due to reaction at residue side chains rather than at the α-amino group. Alanine had been identified previously as the amino-terminal residue of the protein by dansylation in the presence of $NaDodSO_4$ (Weiner et al., 1972). Fragment III thus originates from the amino-terminal end of the protein.

Cleavage of the mutant protein yields the same number of fragments, two of which (I and II) differ from the corresponding fragments of the wild type protein, in that their apparent molecular weights are increased from 22 000 to 24 000 and from 15 000 to 17 000, respectively (Figure 2). On the basis of this analysis, the mutant protein appears to be altered within the region of the protein that contributes fragment II, a fragment that is derived from the interior of the J protein primary structure. This is consistent with genetic analysis of the *hisJ5625* mutation, which indicates that the mutation maps within the interior of the *hisJ*, the gene coding for the structure of the J protein (Noel, 1977).

Tryptic Maps. Trypsin digests of the wild type and mutant proteins were subjected to a two-dimensional separation on thin-layer cellulose (Chen, 1976). Thirty-two peptides are stained by ninhydrin (Figure 4), but no differences were evident between the two proteins. However, phenanthrenequinone staining to locate arginine-containing peptides (Easley et al., 1969) revealed a single difference: the wild type J protein digest has material at the origin that contains arginine (Figure 4), whereas the mutant J* does not.

Since the amino acid composition data suggested that in the mutant protein a cysteine residue had replaced an arginine present in the wild type, the cysteines of both proteins were labeled with radioactive iodoacetic acid. The tryptic map of the labeled mutant J* protein has radioactivity (at the origin of electrophoresis) not present in the map of the labeled wild type J protein (Figure 5). This tryptic peptide which in the mutant contains a cysteine residue instead of the normal arginine was purified from tryptic digests of both proteins. The amino acid compositions of these two peptides are shown in Table III. The compositions are essentially identical except for the substitution of cysteine for arginine in the mutant. It appears that trypsin does not cleave the J protein at this particular arginine residue.

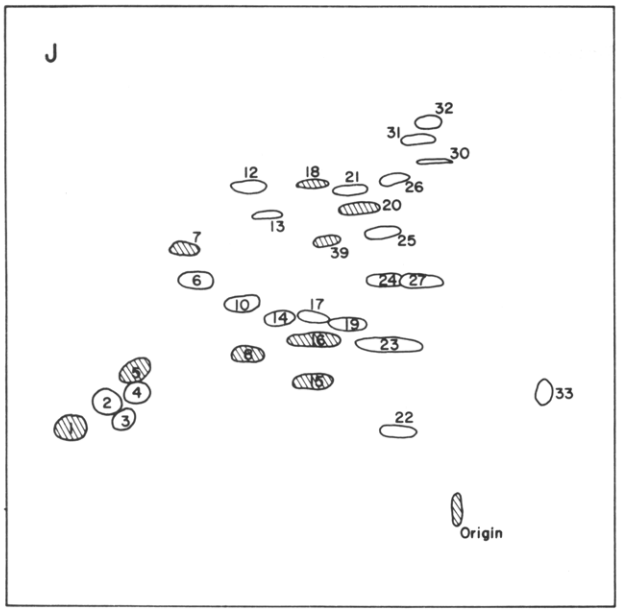

FIGURE 4: Summary of tryptic peptide maps (ninhydrin and arginine-specific staining). These are composite tracings of several tryptic peptide maps on thin-layer cellulose. Only the wild type map is shown. Ninhydrin staining is outlined with the solid line, and arginine-specific staining (with phenanthrenequinone) is represented by the diagonal shading. Electrophoresis is from right to left, and chromatography is from bottom to top. The only difference shown by the mutant protein map is the lack of arginine-specific staining at the origin.

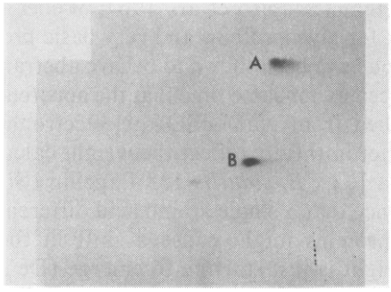

FIGURE 5: Cysteine peptides from tryptic digests. The proteins were labeled with [2-^{3}H]iodoacetic acid, as described under Materials and Methods, and subsequently digested with trypsin. The digest was separated by electrophoresis (right to left) and chromatography (bottom to top) on a thin-layer cellulose plate. After staining for total peptide with ninhydrin, fluorography was performed by the method of Randerath (1970). Only the fluorogram of the wild type digest is shown. The origin is indicated by the dotted line. The mutant protein digest was the same except for additional radioactivity at the origin. Cysteine peptides A and B appear to be peptides 31 and 19 of Figure 4. Evidence of disulfide: only the peptide at the origin is labeled when the mutant protein is reacted with [2-^{3}H]iodoacetic acid, before being chemically reduced. Peptides A and B of mutant and wild type are not labeled under such conditions.

Trimethylaminoethylation of Cysteine Residues. On the basis of analyses to this point, it appeared likely that the only difference between the two proteins was a substitution of a cysteine for an arginine residue. To investigate whether the replacement of an arginine by a cysteine could be responsible for the altered mobility of the mutant protein on an $NaDodSO_4$ gel, we reacted the proteins with (2-bromoethyl)trimethylammonium bromide (BETAB). This reagent alkylates sulfhydryl groups and converts cysteine into L-[2-[(2-amino-2-carboxyethyl)thio]ethyl]trimethylammonium ion, a moiety resembling arginine, in which a quaternary amino group is the analogue of the guanidinium group of arginine (Itano & Robinson, 1972). After the proteins were reduced and treated with BETAB, the migration of the mutant J* protein in $NaDodSO_4$ gel electrophoresis was substantially faster (though not identical with that of the untreated wild type protein), whereas the mobility of the wild type protein was hardly changed by the BETAB treatment (Figure 6).

Table III: Amino Acid Content of the Mutated Tryptic Peptide and Its Wild Type Counterpart

amino acid	wild type	mutant
Cys	0.2[a]	0.9[b]
Asx	5.3	4.7
Thr	1.6	1.0
Ser	2.0	2.1
Glx	(5)	(5)
Gly	3.4	3.1
Ala	4.0	4.6
Val	1.3	1.1
Ile	2.2	1.8
Leu	1.8	1.6
Tyr	0.7	0.9
Phe	1.4	1.7
Lys	1.4	1.1
Arg	1.0	0.1

[a] Average of 4 analyses arbitrarily normalized to 5.0 Glx residues. [b] Average of 5 analyses arbitrarily normalized to 5.0 Glx residues.

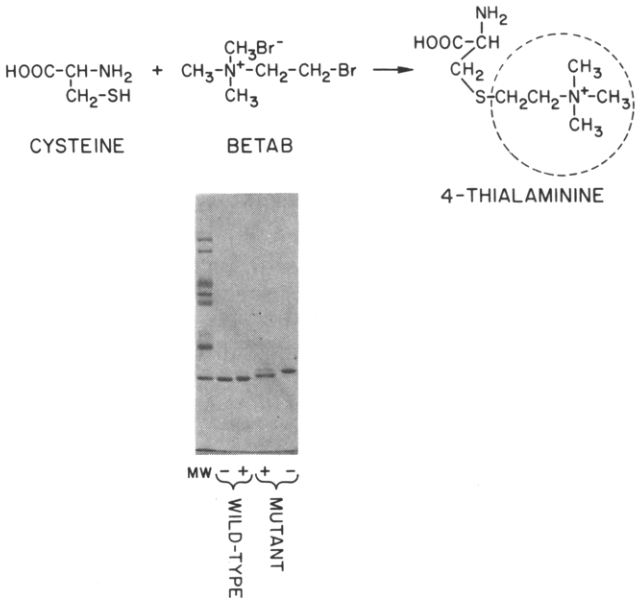

FIGURE 6: Reaction of the J protein with (2-bromoethyl)trimethylammonium bromide (BETAB). The structure of BETAB and the product of its reaction with cysteine are shown above. Below is the $NaDodSO_4$–polyacrylamide gel electrophoretic analysis of trimethylaminoethylated J protein and mutant J* protein: (+) protein reacted with BETAB; (−) no treatment. This is a 10% acrylamide gel run in the Tris–glycine buffer system. The reaction with BETAB was performed as specified by Glazer et al. (1976). (MW) Standard proteins ranging in molecular weight from 25 000 to 130 000.

Reversion of Mutation hisJ5625. One criterion of a missense point mutation is that it can be reverted at a reasonable frequency to the wild type allele. In the case of mutation *hisJ5625*, this criterion is met. One in 10^8 mutant cells reverts to the wild type phenotype [as judged by growth on D-histidine (Kustu & Ames, 1974)], and the J protein from these revertants has the wild type mobility on $NaDodSO_4$–polyacrylamide gel electrophoresis. Thus, the reversion of this mutation is consistent with its being a single base-pair substitution.

Cysteine Residues of the J Protein. The two cysteine residues of the wild type J protein form a disulfide bridge, as shown by the following experiment. The protein was reacted

Table IV: Reaction of Wild Type and Mutant J Proteins with Iodoacetate

treatment	incorpn of carboxymethyl group[a]	
	wild type	mutant
[^{3}H]iodoacetate before redn	1.92×10^4 (5)	1.18×10^5 (32)
[^{3}H]iodoacetate after redn	2.44×10^5 (66)	3.70×10^5 (100)

[a] Counts per minute per milligram of protein. In parentheses is the same value expressed as percent incorporation relative to that of the reduced mutant protein. The procedure for this experiment is described under Materials and Methods. Gdn·HCl (6 M) is present during reduction by β-mercaptoethanol and during reaction with iodoacetate.

with radioactive iodoacetic acid in the presence of 6 M guanidine hydrochloride, before and after reduction by mercaptoethanol. The results are shown in Table IV and Figure 5. Both mutant and wild type proteins have a disulfide bridge, and the same cysteine residues are involved in each case: only the cysteine residue which is unique to the mutant reacts with iodoacetate without prior reduction; the other two cysteines must therefore be oxidized, most likely as a disulfide. Thus, it appears that there is not reshuffling of the sulfhydryl groups in the mutant protein.

The disulfide bond of the wild type J protein is not necessary for its in vitro histidine-binding activity. Preliminary results indicate that alkylation of the reduced protein by iodoacetamide does not drastically decrease the activity.

The extra sulfhydryl group of the mutant protein was shown (Noel, 1977) to be located within CNBr fragment II by cyanylation followed by incubation at alkaline pH (Degani & Patchornik, 1974).

Discussion

The data which have been presented indicate that a wild type arginine residue has been replaced by a cysteine at some site in the interior of the mutant polypeptide chain. Substitution of cysteine for arginine could have been wrought by a single base substitution in the *hisJ* DNA, since the codons for cysteine, UGU and UGC, differ only one base from two of the arginine codons, CGU and CGC. Consistent with a single substitution is the frequency of reversion of this mutation to the wild type. One in 10^8 mutant cells reverts in this manner.

Genetic evidence (Ames & Spudich, 1976) suggests that this mutation has impaired the ability of the histidine-binding protein J to interact with the histidine-transport protein P. It may be that this arginine residue is part of the domain of J protein–P protein contact or in some other way promotes the J protein–P protein interaction, perhaps as a critical element in J protein conformation. However, just as likely is the possibility that arginine is not essential, but replacing it with cysteine is deleterious. Since the binding activity of the mutant protein remains unimpaired, any hypothetical conformational change created by the amino acid replacement must be confined to the domain in which the substitution has occurred.

An initial impetus for studying this mutant protein was its abnormal mobility in $NaDodSO_4$–polyacrylamide gel electrophoresis, which suggested that it had a higher molecular weight than the wild type protein. Because preliminary evidence suggested that a protein modification, such as phosphorylation or glycosylation, was not involved, we examined its amino acid composition (the possible presence of lipid was not investigated). In light of the data which have been presented, it is likely that the difference in electrophoretic migration is due entirely to the substitution of a cysteine for an arginine residue. Evidence that this simple substitution could be responsible is provided by changing this particular cysteine to an arginine-like residue through trimethylaminoethylation. This modification eliminates much of the change in $NaDodSO_4$–polyacrylamide mobility. It is of particular interest that the similar conversion of the other two cysteines of the protein does not have such an effect on $NaDodSO_4$ gel electrophoretic mobility, as shown by the unaltered mobility of the wild type protein after trimethylaminoethylation. Therefore, this is a local $NaDodSO_4$–polypeptide effect due not only to the individual arginine (cysteine) residue but also to the surrounding residues. This effect may be due to increased $NaDodSO_4$ binding when this particular arginine is present (Maley & Guarino, 1977) or to a difference in the conformation of the $NaDodSO_4$–protein complexes, perhaps manifested by greater α-helical character when arginine is present (Mattice et al., 1976) and, as a consequence, a tighter structure. Recently, De Jong et al. (1978) have shown that single amino acid differences in the protein α-crystallin (A chain) can result in $NaDodSO_4$–polyacrylamide electrophoretic mobility changes. In their study, neutral substitutions of hydrophobic residues for hydrophilic ones increased the electrophoretic mobility. The substitution of (cationic) arginine for (neutral) cysteine documented here thus represents another such class.

Presently it is a routine assumption that mobility of a protein in $NaDodSO_4$ gel electrophoresis is governed only by its molecular weight (Shapiro et al., 1967; Weber & Osborn, 1969), except for glycoproteins and very basic proteins (e.g., histones) which have been shown to behave aberrantly. While it has been accepted for some time that the apparent molecular weight obtained from $NaDodSO_4$ gel electrophoresis of a protein may not faithfully reflect the weight calculated from sequence data [e.g., *B. subtilis* 168 flagellin (Simon et al., 1977)], evidence that a single amino acid difference between two related proteins might cause a shift in the apparent molecular weight is just starting to emerge (De Jong et al., 1978). It is now for the first time a simple matter to scan by $NaDodSO_4$ gel electrophoresis numerous mutant proteins. We were able to do so for 66 *hisJ* mutants and found that among those *hisJ* mutants which still produce a J protein, 3 out of 23 (i.e., 13%) yielded a protein with altered mobility (Kustu & Ames, 1974; S. G. Kustu and G. F. Ames, unpublished experiments). Moreover, the mobility alterations were in the direction of both higher and lower apparent molecular weight and by differing extents. Thus, *hisJ* mutations producing a J protein with altered mobility are relatively common. Other workers [e.g., Linn et al. (1975), Funatsu et al. (1972), Buchanon & Strominger (1976), and Wu et al. (1977)] have observed mutant proteins whose $NaDodSO_4$ gel electrophoretic behavior differed from the wild-type behavior, but to our knowledge in no other case has a chemical analysis been achieved which established whether or not the mutation actually produced a change in the molecular weight of the protein.

In conclusion, it is clear that the overall amino acid composition of a protein can affect its mobility in $NaDodSO_4$ gel electrophoresis (Simon et al., 1977), and so can covalent modification (P. C. Billings, J. W. Orf, D. A. Talmage, and M. Blumenfeld, unpublished experiments). Here we show that the mutational substitution of a single amino acid can also do so. In light of these findings, we feel that altered proteins cannot be assumed to have a different molecular weight, if the interpretation is based only on a $NaDodSO_4$ gel electrophoretic

mobility change, and that additional alternative evidence should be sought.

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Thallous Ion Is Accumulated by Potassium Transport Systems in *Escherichia coli*†

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ABSTRACT: The accumulation of $^{204}Tl^+$ by *Escherichia coli* occurs primarily via either of two K^+ transport systems called Kdp and TrkA. Tl^+ influx is inhibited and Tl^+ efflux is stimulated by the addition of K^+ to the assay medium. Mutants defective in both the Kdp and TrkA systems accumulate little Tl^+. Uptake of triphenylmethylphosphonium, a lipid-soluble cation whose distribution is widely used to estimate the membrane electrical potential in bacteria, occurs to about the same extent in mutants that accumulate little Tl^+ as in strains that accumulate Tl^+ to high levels. These findings indicate that Tl^+ may be useful as a probe of bacterial K^+ transport systems but is not a reliable indicator of the membrane electrical potential in *E. coli*.

Interpretation of membrane-related phenomena in chemiosmotic terms requires a knowledge both of the concentration ratio of the ions or molecules involved and of the transmembrane electrical potential, $\Delta\psi$.[1] This potential can be estimated directly with microelectrodes in large cells, but in small cells or organelles indirect methods must be used

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[1] Abbreviations used: $\Delta\psi$, electrical potential difference across the cytoplasmic membrane (interior negative); Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, Tris(hydroxymethyl)aminomethane; EDTA, (ethylenedinitrilo)tetraacetic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; $TPMP^+$, triphenylmethylphosphonium; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone.

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