Subunit Structure of Islet-Activating Protein, Pertussis Toxin, in Conformity with the A–B Model†

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ABSTRACT: The subunit structure of islet-activating protein (IAP), pertussis toxin, has been analyzed to study a possibility that this protein is one of the A–B toxins [Gill, D. M. (1978) in *Bacterial Toxins and Cell Membranes* (Jeljaszewicz, J., & Wadstrom, T., Eds.) pp 291–332, Academic Press, New York]. Heating IAP with 1% sodium dodecyl sulfate caused its dissociation into five dissimilar subunits named S-1 (with a molecular weight of 28 000), S-2 (23 000), S-3 (22 000), S-4 (11 700), and S-5 (9300), as revealed by polyacrylamide gel electrophoresis; their molar ratio in the native IAP was 1:1:1:2:1. The molecular weight of IAP estimated by equilibrium ultracentrifugation was 117 000 which was not at variance with the value obtained by summing up molecular weights of the constituent subunits. The preparative separation of these IAP subunits was next undertaken; exposure of IAP to 5 M ice-cold urea for 4 days followed by column chromatography with carboxymethyl-Sepharose caused sharp separation of S-1 and S-5, leaving the other subunits as two dimers. These dimers were then dissociated into their constituent subunits, i.e., S-2 and S-4 for one dimer and S-3 and S-4 for the other, after 16-h exposure to 8 M urea; these subunits were obtained individually upon further chromatography on a diethylaminoethyl-Sepharose column. Subunits other than S-1 were adsorbed as a pentamer by a column using haptoglobin as an affinity adsorbent. The same pentamer was obtained by adding S-5 to the mixture of two dimers. Neither this pentamer nor other oligomers (or protomers) exhibited biological activity in vivo. Recombination of S-1 with the pentamer at the 1:1 molar ratio yielded a hexamer which was identical with the native IAP in electrophoretic mobility and biological activity to enhance glucose-induced insulin secretion when injected into rats. In the broken-cell preparation, S-1 was biologically as effective as the native IAP; both catalyzed ADP-ribosylation of a protein in membrane preparations from rat C6 glioma cells. In conclusion, IAP is an oligomeric protein consisting of an A (active) protomer (the biggest subunit) and a B (binding) oligomer which is produced by connecting two dimers by the smallest subunit in a noncovalent manner. Rationale for this terminology is discussed based on the A–B model.

Islet-activating protein (IAP)[1] is one of the pertussis toxins (Munoz & Bergman, 1978; Pittman, 1979). It was isolated from the culture medium of *Bordetella pertussis* (Yajima et al., 1978a,b; Ui et al., 1978) based on the findings that an injection of pertussis vaccine into rats caused sustained potentiation of insulin secretion from their pancreatic islets in response to various stimuli (Sumi & Ui, 1975; Katada & Ui, 1977, 1979b). Later studies revealed that the toxin acts on not only islet cells but also other cell types including rat heart and C6 glioma cells; receptor-mediated changes in the cellular content of cyclic AMP, or in adenylate cyclase activity, were markedly modified by prior exposure in vivo or in vitro of these cells to IAP (Katada & Ui, 1979a, 1981a,b; Hazeki & Ui, 1981; Katada et al., 1982). The latest reports (Katada & Ui, 1982a,b) have shown that IAP catalyzes transfer of an ADP-ribose moiety from NAD to one of the guanine nucleotide regulatory components in the receptor–adenylate cyclase system, thereby enhancing activation of receptor-linked and GTP-dependent adenylate cyclase. Thus, IAP resembles cholera (Moss & Vaughan, 1979) and diphtheria (Hayaishi & Ueda, 1977) toxins in catalyzing ADP-ribosylation of guanine nucleotide-binding proteins.

An "A–B model" has been proposed for several toxic peptides that cross cell membranes (Gill, 1978); any of these peptides, including cholera and diphtheria toxins, consists of two functionally distinct parts, an A component that is active enzymatically and a B component that binds to surface receptors to enable the A component to enter the cell where it acts. The interaction of these toxins with intact cells is characterized by a lag time that precedes the onset of their action, probably a time required for the A component to traverse the plasma membrane. Such was the case with IAP; e.g., there was a definite lag period of 1 h before α-adrenergic inhibition of insulin secretion started to be reversed progressively in islet cultures with IAP (Katada & Ui, 1980). IAP was previously reported to be an oligomeric protein comprising dissimilar peptides (Kanbayashi et al., 1978). Possibly, the molecular structure of the pertussis toxin may also conform to the A–B model, with an A protomer separable or dissociable from others which, in combination, may act as the B oligomer just as has been reported for cholera toxin.

The purpose of the present paper is to examine this possibility by undertaking (a) analysis of the subunit structure of IAP, (b) isolation of the individual subunits, and (c) their stepwise assembly to reconstitute the native IAP eventually. It is shown that the native IAP consists of an A protomer and a B oligomer that is an association product of two dimers by means of an additional connecting subunit. A one-step dissociation of the whole toxin to the A protomer and the B oligomer was feasible by means of affinity chromatography using haptoglobin as an adsorbent of the B oligomer. The A protomer was as effective as the native IAP in catalyzing ADP-ribosylation of the M_r 41 000 protein of membranes of rat C6 glioma cells.

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[1] Abbreviations: IAP, islet-activating protein; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; CM, carboxymethyl.

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Materials and Methods

Materials. IAP was purified from the 2-day culture supernatant of *B. pertussis* (Tohama strain, phase I) according to the procedure previously reported (Yajima et al., 1978a). CM-Sepharose, DEAE-Sepharose, Sephacryl S-200, CNBr-activated Sepharose 4B, Ficoll 400, and Pharmalyte (pH 3.5–10.0) were the products from Pharmacia Fine Chemicals. Purified human haptoglobin was obtained from Green Cross Corp. (Osaka). The haptoglobin–Sepharose 4B affinity adsorbent was prepared according to the procedure of Irons & MacLennan (1979). The reagents for radioimmunoassay of plasma insulin were obtained from Dainabot Radioisotope Laboratories (Tokyo), and rat insulin (Novo) used as standard was a kind gift from Kodama Company Ltd. (Tokyo). [α-^{32}P]NAD (29 Ci/mmol) was purchased from New England Nuclear. Other reagents were of analytical grade from commercial sources.

Assay of Biological Activity in Vivo. The biological activity of IAP and its components was assayed on the basis of their activity to enhance the insulin secretory response of rats to glucose load (Yajima et al., 1978a). Male Wistar rats weighing 120–150 g were injected intravenously with the sample solution. Saline was injected instead into control rats. On the third postinjection day, these rats (20-h fasted) were further injected intraperitoneally with a 20% solution of glucose (1 mL/100 g of body weight). From the increases in plasma insulin (ΔIRI) and blood glucose (ΔGlc) during the first 15 min after glucose load, an insulinogenic index ($R = \Delta IRI/\Delta Glc$) was calculated for each rat. The IAP activity unit is then defined as $(R_t - R_c)/R_c \times 100$, where R_t and R_c are the indexes of treated and control rats, respectively. Three treated and three control rats were used for calculation of the IAP activity of one sample.

Electrophoresis. Different electrophoretic procedures were applied: (a) separation of subunits (Figures 1, 3, 5, and 8), (b) determination of their molecular weights, (c) estimation of a molar ratio of subunits in the IAP molecule, (d) subunit assembly analysis (Figure 6), and (e) assessment of isoelectric points of subunits (Table I). The eluate from chromatographic columns had been concentrated by means of Ficoll 400 before being applied to electrophoresis. For procedures a, b, and c where electrophoresis was carried out in the presence of 0.1% $NaDodSO_4$, the protein samples had been incubated with 1% $NaDodSO_4$ and 4 M urea for 5 min at 100 °C. Procedure a was performed at 120 V for 14 h in the discontinuous slab gel system as described by Laemmli (1970) with slight modifications. The acrylamide concentration in a running gel was increased from 10 (top) to 30% (bottom) with a constant (37/1) acrylamide/*N*,*N*′-methylenebis(acrylamide) ratio. The running gel (11 cm), stacking gel (1.5 cm, 4% acrylamide), and chamber solution contained 0.375 M Tris-HCl (pH 8.8), 0.129 M Tris-HCl (pH 6.8), and 0.025 M Tris plus 0.129 M glycine (pH 8.8), respectively. In the case of procedure c, the running gel was prepared by mixing 10% acrylamide with decreasing concentrations of *N*,*N*′-methylenebis(acrylamide) [the ratio, from 37/1 (top) to 10/1 (bottom)] and increasing concentrations of urea from 1.5 to 8 M. After 5-h electrophoresis at 125 V, the gel was destained and scanned densitometrically at 550/480 nm with a Shimadzu CS-900 double wavelength TLC scanner. A constant amount (5 μg) of the subunit S-3 was used as an internal standard for densitometric quantitation.

Electrophoresis for procedure b was conducted in 12.5% polyacrylamide gel rods for 7 h by the method of Weber & Osborn (1969). The constant electric current of 8 mA/rod was applied at 4 °C. Molecular weight markers used were ovalbumin (M_r 45 000), yeast alcohol dehydrogenase (35 000), chymotrypsinogen (25 000), myoglobin (17 200), ribonuclease A (13 200), cytochrome *c* (12 300), egg white lysozyme (10 400), proinsulin (9300), and insulin (5700).

For procedure d, protein samples were electrophoresed for 3 h in 7.5% polyacrylamide gel rods at pH 4.0 or 8.9 by the method of Reisfeld & Lewis (1962). The electric current of 1 mA/rod was applied at 4 °C. Isoelectric focusing (e) was performed in 4% polyacrylamide gels containing 8.5 M urea, 2% Nonidate P-40, and 2% Pharmalyte (pH 3.5–10.0) (O'-Farrell, 1975). A constant voltage (300 V) was applied for 15 h at 4 °C. After electrophoresis, the gel was repeatedly washed to remove Pharmalyte and was cut into 5-mm sections each of which was soaked in 2 mL of water to estimate the pH. The protein bands were detected by staining gels in the parallel run.

All the electrophoretic gels were stained with Coomassie Brilliant Blue R-250 except for procedure d where Amido Black 10B was used for staining protein bands.

Assay of ADP-Ribosyltransferase Activity. This enzyme activity of IAP and its dissociation products was assayed by determining the incorporation of an [^{32}P]ADP-ribose moiety from [α-^{32}P]NAD into the membrane protein of rat C6 glioma cells as described elsewhere (Katada & Ui, 1982a,b). Briefly, crude membranes (200 μg of protein) prepared from C6 cells were incubated with IAP (25 μg/mL), A protomer (6.5 μg/mL), or B oligomer (18.5 μg/mL) for 10 min in 100 μL of the medium containing 25 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 1 mM ATP, 10 mM thymidine, and 10 μM [α-^{32}P]-NAD (10 Ci/mmol). Membranes thus radiolabeled were dissolved in a gel sample buffer (1% $NaDodSO_4$, 5% 2-mercaptoethanol, 10% glycerol, 62.5 mM Tris-HCl, and 0.02% bromophenol blue, pH 6.8) and heated for 3 min at 100 °C. Aliquots (50 μg of protein) were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis on slab gels which consist of a 12.5% separating gel and a 4.5% stacking gel. After electrophoresis, gels were stained as described above, destained, dried, and exposed to Kodak X-Omat film for 24 h at −80 °C.

Analytical Ultracentrifugation. Sedimentation–velocity experiments were performed at 60 000 rpm and 11.5 °C in phosphate buffer at various pHs with the use of a Hitachi UCA-1 analytical ultracentrifuge equipped with a UV scanner optical system. The phosphate buffer was invariably supplemented with 2 M urea. The experimental data were analyzed by the method of boundary analysis (Van Holde & Weischet, 1978). High-speed sedimentation equilibrium experiments were carried out by the method of Yphantis (1964), which was modified for multicomponent analysis based on successive equilibria at two rotor speeds, using a Hitachi analytical ultracentrifuge. Calculations were made with a partial specific volume of 0.728 for native IAP which was determined from its amino acid compositions.

Amino Acid and Protein Analyses. Protein samples were hydrolyzed in evacuated tubes in 6 N HCl at 110 °C for 48 h; the hydrolysate was applied to a Hitachi 835-50 amino acid analyzer. The protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Results

Subunit Structure of IAP. Heating of IAP in a 1% $NaDodSO_4$ and 4 M urea solution at 100 °C for 5 min gave rise to disintegration of the toxin molecule into five dissimilar peptides as revealed by polyacrylamide gel electrophoresis (Figure 1). These peptides (subunits) will be henceforth

FIGURE 1: Separation of IAP subunits by $NaDodSO_4$–polyacrylamide gel electrophoresis. IAP (20 μg) was submitted to electrophoresis as described for procedure a under Materials and Methods.

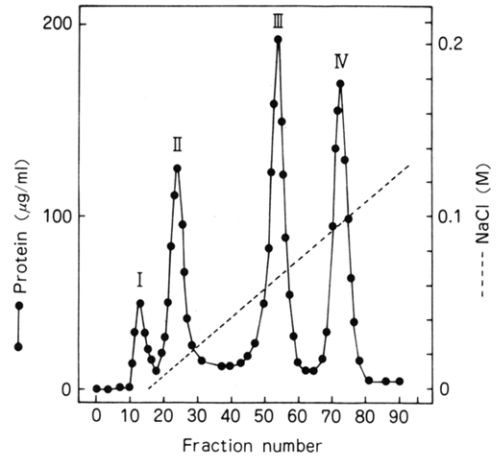

FIGURE 2: CM-Sepharose chromatography of IAP partially dissociated in 5 M urea. IAP (18 mg) was dissolved in 3 mL of 0.05 M phosphate buffer (pH 6.0) containing 5 M urea and stored at 4 °C for 4 days before being applied to a column (1.5 × 21 cm) of CM-Sepharose CL-6B previously equilibrated with the same urea-containing buffer. The column was eluted with 400-mL linear gradients of 0–0.25 M NaCl and 0.05 (pH 6.0)–0.1 (pH 7.0) M phosphate buffer in 5 M urea. The flow rate was 25 mL/h, and the fraction volume was 3 mL.

referred to as S-1, S-2, S-3, S-4, and S-5 in the reversed order of their electrophoretic mobilities. Their respective molecular weights were estimated to be 28 000, 23 000, 22 000, 11 700, and 9300 by $NaDodSO_4$–polyacrylamide gel electrophoresis, based on a calibration curve obtained with nine proteins with known molecular weights as described under Materials and Methods.

Isolation and Purification of Subunits. IAP is stable in 2 M urea at pH 7.0, a vehicle usually employed for the stock solution, but its biological activity was lost by increasing the concentration of urea, probably due to its dissociation into smaller peptides. Taking advantage of such instability in concentrated urea, IAP stored in 5 M urea (pH 6.0) at 4 °C for 4 days was applied to a column of CM-Sepharose CL-6B. Four kinds of peptides were then eluted one after another (peaks I–IV) from the column with linear gradients of pH and ionic strength (Figure 2). Peak III was rechromatographed under the same conditions to be freed of minute contamination with whole IAP; each of the four peaks then migrated as a single and symmetrical band during gel electrophoresis as described for procedure d under Materials and Methods (data not shown). Electrophoretic analysis in the presence of $NaDodSO_4$ revealed that peaks I and II were identical with S-5 and S-1, respectively (Figure 3B,C). Peaks III and IV were both oligomer; the former was composed of S-3 and S-4 (Figure 3D) while the latter was an aggregate of S-2 and S-4 (Figure 3E). They should be dimer, because their molecular weight was less than one-third the molecular weight (117 000; see below) of native IAP (e.g., see Figure 9, inset). Proteins eluted as peaks III and IV are henceforth referred to as dimer 2 and dimer 1, respectively.

Further dissociation of dimer 1 and dimer 2 into their constituent peptides was carried out in 8 M urea solution (pH 8.4) for 16 h at 4 °C. The solution was then chromatographed on a DEAE-Sepharose column with the same urea solution (Figure 4). Dimer 1 split into S-2 and S-4 (Figure 4B) while dimer 2 was separated into S-3 and S-4 (Figure 4A), as evidenced in Figure 5B–E. The chromatographic (Figure 4) and electrophoretic (Figure 5D,E) behavior of S-4 originating from dimer 1 was indistinguishable from that from dimer 2.

Amino Acid Composition and Isoelectric Points of Native IAP and Its Subunits. Subunits isolated and purified as described above were analyzed for their amino acid compositions and isoelectric points in comparison with those of the native IAP (Table I). The amino acid content and isoelectric point

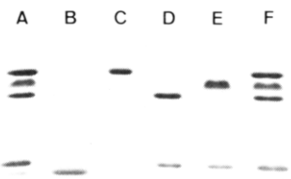

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of dissociation products of IAP. Fractions separated in Figure 2 were electrophoresed as described for procedure a under Materials and Methods. Fractions applied and their protein amounts are the following: lanes A and F, native IAP (20 μg); lane B, peak I (8 μg); lane C, peak II (4 μg); lane D, peak III (8 μg); lane E, peak IV (8 μg).

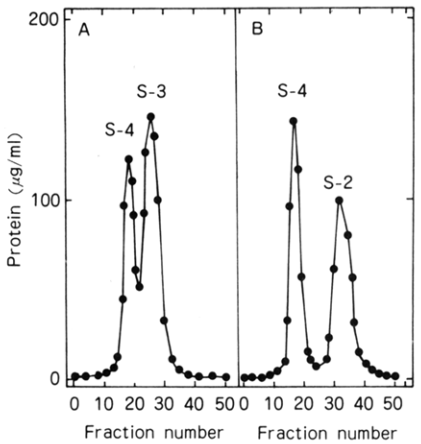

FIGURE 4: Elution profiles of dissociation products of dimer 1 and dimer 2 from a DEAE-Sepharose column. Peaks III (panel A) and IV (panel B) obtained in Figure 2 were concentrated to 2 mL (both 2.5 mg of protein) by means of Ficoll 400 and dialyzed against 1 L of 0.017 M phosphate buffer (pH 8.4) containing 8 M urea for 16 h at 4 °C before being applied to a column (1.9 × 42 cm) of DEAE-Sepharose CL-6B. The column was eluted with the same buffer at a flow rate of 15 mL/h. The fraction volume was 1.8 mL.

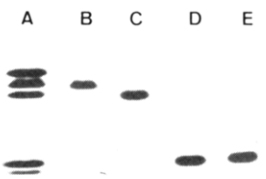

FIGURE 5: $NaDodSO_4$–polyacrylamide gel electrophoresis of dissociation products of dimer 1 and dimer 2. Fractions (5 μg of the protein each) obtained in Figure 4 were electrophoresed as described for procedure a under Materials and Methods. Lane A, native IAP (25 μg); lane B, the second peak for dimer 1 (8 μg); lane C, the second peak for dimer 2 (8 μg); lane D, the first peak for dimer 1 (8 μg); lane E, the first peak for dimer 2 (8 μg).

Table I: Amino Acid Composition and Isoelectric Point (pH) of IAP and Its Subunits

	residues (% of total)						
	IAP	S-1	S-2	S-3	S-4[a]	S-4[b]	S-5
Asp	7.5	9.3	6.3	6.3	5.3	5.0	8.2
Thr	7.4	7.4	10.4	8.2	5.0	5.1	6.9
Ser	7.3	10.6	8.5	6.3	8.0	7.3	6.9
Glu	9.0	10.6	8.7	9.0	9.5	9.1	9.3
Gly	9.6	11.2	13.0	11.9	9.6	8.9	8.7
Ala	10.0	10.6	6.5	11.7	9.4	9.8	9.8
Cys	1.2	1.0	1.3	1.1	0.9	0.7	1.6
Val	6.7	6.7	4.9	4.7	9.4	9.4	4.0
Met	2.5	1.6	1.5	1.1	5.1	4.3	1.6
Ile	3.6	3.2	4.2	5.0	2.0	1.8	3.4
Leu	7.9	5.5	7.3	8.1	8.4	8.7	13.8
Tyr	6.1	4.6	7.6	7.9	2.2	2.0	4.3
Phe	3.9	3.5	3.2	3.2	3.6	4.5	4.9
Lys	3.7	2.2	3.4	2.7	6.9	7.6	4.7
His	1.2	1.7	2.4	1.0	0.5	0.5	3.0
Arg	6.3	5.9	6.2	6.1	5.1	5.4	3.3
Pro	6.1	4.4	4.6	5.7	9.1	9.9	5.6
pH		5.8	8.5	8.8	10	10	5.0

[a] Originating from dimer 1. [b] Originating from dimer 2.

for S-4 were essentially the same regardless from which dimer it originated. The most acidic peptide was S-5. S-2 and S-3 were similar to each other in isoelectric point and amino acid composition except for the content of alanine; hence, two dimers were also similar (data not shown).

Molar Ratio of Subunits in the IAP Molecule. Five subunits were applied, separately and in various amounts, to $NaDodSO_4$ gel electrophoresis followed by densitometry as described for procedure c under Materials and Methods. The color intensity of the stained band on gel was proportional to the amount (60–600 pmol) of the subunit applied, providing a linear calibration curve for each subunit (correlation coefficients were between 0.964 and 0.984 for the five subunits). The native IAP was also applied to gel electrophoresis under the same conditions to assess, on the basis of these calibration curves, the molar ratio of the separated five subunit bands. It was 0.88 (S-1)/0.93 (S-2)/1.02 (S-3)/2.25 (S-4)/1.00 (S-5) as an average of duplicate observations.

Molecular Weight of IAP. Molecular weights and molar contents of the constituent subunits as determined above gave a calculation value of 106 000 for the molecular weight of their association product, native IAP. This is different considerably from the value of 77 000 previously reported based on a gel filtration experiment with Sephadex (Yajima et al., 1978a). Even a smaller value was obtained if Sephadex was replaced by Sephacryl (e.g., see Figure 9, inset). Since we had later noticed that IAP tended to be somewhat adsorbed by Sephadex and Sephacryl, leading to an underestimation of molecular weight, equilibrium ultracentrifugation of an IAP solution was undertaken to assess its molecular weight more accurately.

IAP is not sufficiently soluble or tends to form insoluble aggregates in distilled water; the addition of 2 M urea was necessary to keep an IAP solution apparently homogeneous. In order to establish the conditions under which no dissociation takes place during ultracentrifugation in 2 M urea, we analyzed sedimenting boundaries at various pHs according to the method of Van Holde & Weischet (1978). There were multiple smaller fragments in the urea solution when the pH was raised up to 9.08 (data not shown). In the 2 M urea solution at slightly acidic pHs, however, IAP was essentially homogeneous with the sedimentation coefficient of 6.6 S (data not shown).

The molecular weight of IAP was then determined by the high-speed sedimentation equilibrium method of Yphantis (1964) in 0.1 M NaCl containing 0.01 M KH_2PO_4 and 2 M urea (pH 4.8). The protein solution with an A_{280} value of 0.3 was centrifuged at the rotor speed of 19 680 or 24 870 rpm at 20.1 °C. The weight-average molecular weight thus obtained was 117 000. Thus, IAP is a hexamer, 1 mol of which consists of 1 mol of S-1, S-2, S-3, and S-5 each and 2 mol of S-4.

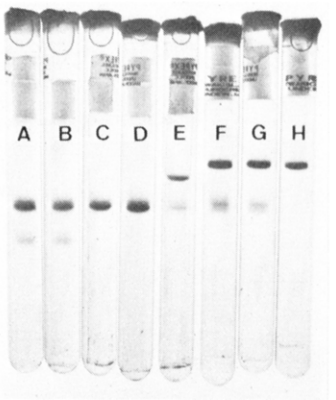

FIGURE 6: Polyacrylamide gel disc electrophoresis of various combinations of IAP subunits. The mixture of subunits (30 μg of protein) prepared as described in Table II was subjected to electrophoresis at pH 4.0 as described for procedure d under Materials and Methods. The subunit combination in lanes A–H is shown in numbers 1–6, 8, and 11 in Table II.

Assembly of Subunits To Reconstitute the Biologically Active IAP. The isolated subunits were combined in 2 M urea to study which combination can afford an association product. As had been expected from the aforementioned isolation of two kinds of dimer upon dissociation of IAP in 5 M urea (Figure 3), the combination of S-4 with S-2 or S-3 produced the association product which was identical with dimer 1 or dimer 2, respectively (Figure 6A,B). No other pair of subunits generated dimer; both of the paired subunits migrated on the gel as two separated bands (data not shown). Likewise, a mixture of three or four subunits in any possible combination gave no single band on gel electrophoresis; when the mixture contained the combination of S-4 with S-2 or S-3, the band corresponding to dimer 1 or dimer 2 was observed, but other subunits migrated on the gel individually (data not shown). Moreover, the mixture of dimer 1 with dimer 2 gave no association product (Figure 6C). Thus, neither trimer nor tetramer was generated at all.

The further addition of S-1 to the mixture of dimer 1 and dimer 2 failed to produce any bigger association product (Figure 6D), whereas the replacement of S-5 for S-1 in the same mixture gave rise to a new protein band with a larger molecular weight (Figure 6E). The addition of S-2, S-3, or S-4 instead of S-5 was without effect in this regard. Thus, the newly formed pentamer resulted from connection of dimer 1 and dimer 2 by means of S-5. The pentamer and S-5 will be hence referred to as the B oligomer and the C (connecting) subunit, respectively (see Discussion). Further addition of S-1 to the B oligomer was effective to yield the protein band (Figure 6F) that migrated on a polyacrylamide gel at the same rate as the native IAP (Figure 6H). The mixture of all the subunits at the same molar ratio as that in the native IAP also migrated on the gel as almost a single band corresponding to the holotoxin (Figure 6G).

Biological activity of the subunit combinations was studied in vivo based on their enhancement of insulin secretory responses of rats to glucose load (Table II). None of the two dimers, alone or in combination, exhibited biological activity;

Table II: Biological Activity of Combinations of IAP Subunits

no.	combinations[a]	IAP act. (units × 10^{-2})
1	S-2 + S-4 (A)[b]	0.6
2	S-3 + S-4 (B)	0.8
3	dimer 1 + dimer 2 (C)	0.5
4	(3) + S-1 (D)	1.0
5	(3) + S-5 (E)	1.1
6	(5) + S-1 (F)	10.4
7	(3) + S-1 + S-5	11.0
8	S-1 + S-2 + S-3 + S-4[c] + S-4[d] + S-5 (G)	10.9
9	S-1 + S-2 + S-3 + S-4[c] + S-4[c] + S-5	9.8
10	S-1 + S-2 + S-3 + S-4[d] + S-4[d] + S-5	9.8
11	IAP (H)	10.6

[a] Before being combined, subunits and dimers were dialyzed against 0.1 M phosphate buffer (pH 7.0) containing 2 M urea for 24 h, and the precipitate, if formed, was removed by centrifugation. The amount (micrograms in 0.1–0.2 mL) used for combination was the following: S-1, 56; S-2, 46; S-3, 44; S-4, 24; S-5, 18; dimer 1, 75; dimer 2, 68. The combined mixture was incubated at 7 °C for 14 h before being applied to electrophoresis (Figure 6) or being injected into rats at a dose of 3 (no. 1–10) or 1 μg (no. 11). The IAP activity unit was determined as described under Materials and Methods. [b] A–H correspond to the lanes in the electrophoretogram in Figure 6. [c] Originating from dimer 1. [d] Originating from dimer 2.

nor was the B oligomer effective on insulin secretion. Likewise, the mixture of four subunits in any possible combination failed to display the IAP activity. The biological activity was recovered when a B oligomer was mixed with S-1 (Table II, line 6). Since this recovery of the IAP activity suggests that the biologically active center may exist in the S-1 peptide (see below), S-1 will be referred to as an A protomer; IAP is thus an oligomeric protein consisting of an A protomer and a B oligomer. The reconstitution of the IAP molecule by adding the constituent subunits one after another or by adding a C subunit and an A protomer at once to the mixture of dimer 1 and dimer 2 afforded the same full biological activity as the one-step combination of an A protomer with a B oligomer. S-4 was equally effective in recovering the IAP activity regardless of whether it originated from dimer 1 or dimer 2 (Table II, lines 8–10).

One-Step Dissociation of IAP to A Protomer and B Oligomer by Affinity Chromatography. The foregoing results are considered to indicate that a whole IAP molecule, in 5 M urea, is first dissociated to an A protomer and a B oligomer which is, in turn, split into two dimers and a C subunit. One-step separation of the A protomer from the B oligomer was therefore undertaken after a short-term exposure of IAP to 5 M urea. For this purpose, advantage was taken of the specific interaction of pertussis toxin with human haptoglobin (Irons & MacLennan, 1979); our preliminary experiments showed that not only IAP but also its B oligomer and dimers 1 and 2 were, but the A protomer and the C subunit were not, adsorbed by haptoglobin–Sepharose.

IAP was exposed to 5 M urea for 6 h at 4 °C and applied to a column of haptoglobin–Sepharose as shown in Figure 7. The protein not bound by the affinity column (Figure 7A) proved to be mostly an A protomer which was slightly contaminated with other subunits (Figure 8A). The electrophoretically homogeneous A protomer was readily obtained by applying this protein fraction to a column (1.6 × 28 cm) of DEAE-Sepharose CL-6B followed by elution with the same 5 M urea containing buffer (Figure 8B).

The protein retained by the adsorbent was eluted by a stepwise change of the buffer to 0.5 M NaCl/0.1 M Tris

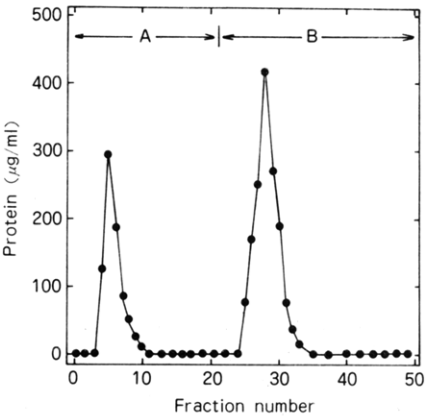

FIGURE 7: Separation of A protomer from B oligomer by affinity chromatography. The IAP solution (10 mg/3 mL) was dialyzed against 1 L of 0.017 M phosphate buffer (pH 8.3) containing 5 M urea for 6 h at 4 °C and applied to a column (1.6 × 30 cm) of haptoglobin–Sepharose 4B that had been equilibrated with the same buffer. The column was eluted first with the same buffer (A) and then with 0.5 M NaCl/0.1 M Tris (pH 10.0) containing 3 M KSCN (B). The flow rate was 30 mL/h, and the fraction volume was 3 mL.

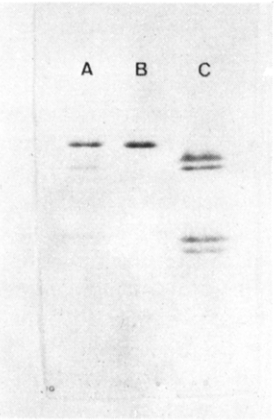

FIGURE 8: $NaDodSO_4$–polyacrylamide gel electrophoresis of A protomer and B oligomer prepared by affinity chromatography. Electrophoresis was conducted as described for procedure a under Materials and Methods. The protein applied to electrophoresis is the following: Lane A, peak A (3 μg) obtained in Figure 7; lane B, peak A (3 μg) obtained in Figure 7 after further purification by column chromatography as described in the text; lane C, the eluate (9 μg) obtained in Figure 9.

solution (pH 10.0) containing 3 M KSCN (Figure 7B). Polyacrylamide gel disc electrophoresis revealed that the eluted protein was the B oligomer which was occasionally contaminated with trace amounts of undissociated IAP. The B oligomer thus prepared and simply purified by gel filtration through a column of Sephacryl S-200 (Figure 9) had a molecular weight slightly smaller than whole IAP (Figure 9, inset) and was composed of all IAP subunits other than S-1 (A protomer), as expected (Figure 8C).

ADP-Ribosyltransferase Activity of IAP and A Protomer. Katada & Ui (1982a,b) recently reported that IAP catalyzed ADP-ribosylation of a M_r 41 000 protein which was one of the subunits of the guanine nucleotide regulatory protein in the receptor–adenylate cyclase system of membranes of rat C6 glioma cells. Figure 10 reproduces their reports by showing that the M_r 41 000 protein was radiolabeled by [α-^{32}P]NAD when the cell-free membrane preparation from C6 cells was incubated with IAP. The A protomer was as effective as IAP, but the B oligomer was without effect, in this regard, indicating that there was an enzymatically active center in the molecule of the A protomer.

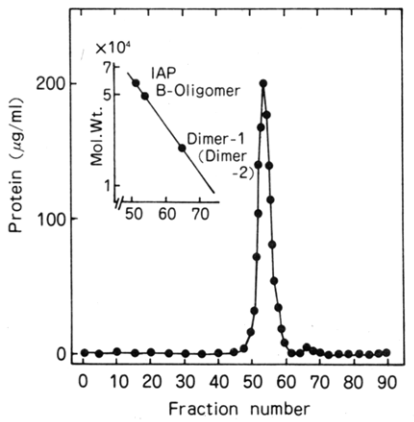

FIGURE 9: Gel filtration of B oligomer prepared by affinity chromatography. Peak B (5 mg of protein) in Figure 7 was concentrated by means of Ficoll 400 and applied to a column (1.6 × 105 cm) of Sephacryl S-200 that had been equilibrated with 0.5 M NaCl/0.1 M phosphate buffer (pH 7.0) containing 2 M urea. The column was eluted with the same buffer at the rate of 20 mL/h with the fraction volume of 2.8 mL. The apparent molecular weight of thus eluted B oligomer is compared with those of native IAP and dimer 1 (dimer 2) in the inset.

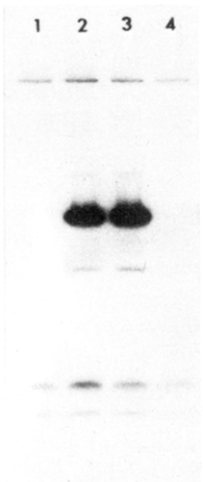

FIGURE 10: IAP-catalyzed or A oligomer catalyzed ADP-ribosylation of an M_r 41 000 protein of membranes from rat C6 glioma cells. Membranes were incubated with [α-^{32}P]NAD in the absence (lane 1) or presence of IAP (lane 2), A protomer (lane 3), or B oligomer (lane 4), and radiolabeled proteins were separated by electrophoresis, as described under Materials and Methods. The main band shown in lanes 2 and 3 corresponds to the protein with an M_r value of 41 000.

Discussion

Bordetella pertussis bacteria produce exotoxins that have been named a histamine-sensitizing factor (HSF) and a leukocytosis (or lymphocytosis)-promoting factor (LPF), based on their biological activities, besides IAP. A single name, pertussigen (Munoz & Bergman, 1978) or pertussis toxin (Pittman, 1979), has been proposed for these proteins, since any of these apparently distinct biological activities, i.e., histamine sensitizing, leukocytosis promoting, and insulin secretion stimulating, was not specific to any one, but was shared by all, of these three kinds of protein. With regard to their subunit structures, exposure of whole toxins to $NaDodSO_4$ was reported to cause their separation to four different peptides for leukocytosis-promoting factor (Morse & Morse, 1976), for hemagglutinin LPF (Arai & Sato, 1976), and for IAP (Kanbayashi et al., 1978). Thus, the present paper is the first to show that pertussis toxin is a hexamer consisting of five dissimilar peptides. Experimental evidence is as follows.

First, application of the IAP solution previously heated with 1% $NaDodSO_4$ and 4 M urea to $NaDodSO_4$–polyacrylamide gel electrophoresis in the discontinuous system gave five bands of peptides sharply separated from each other (Figure 1). The addition of 2-mercaptoethanol before heating resulted in the same electrophoretogram, excluding a possibility that there are S–S bonds linking peptide chains. The discrepancy between the previous report by Kanbayashi et al. (1978) and the present one may be largely related to the difference in the concentration of gel used; when electrophoresis was repeated with 10% polyacrylamide gel as used by Kanbayashi et al. (1978), no separation occurred between the bands corresponding to S-2 and S-3, and a band for S-5 was too faint in color to be evidently detected (data not shown). This may be the reason why Kanbayashi et al. (1978) found only three protein bands upon electrophoresis.

Second, exposure of IAP to 5 M urea for a period as long as 4 days caused its dissociation into two monomers, S-1 and S-5, and two dimers, dimer 1 and dimer 2, which were further split into their constituent monomers (S-2 plus S-4 and S-3 plus S-4, respectively) after the subsequent exposure to 8 M urea. Thus, IAP should be an association product of two monomers and two dimers: a hexamer. This is in good agreement with the molar ratio of subunits estimated from their relative color intensity on the electrophoretogram; the molar ratio of S-4, a common constituent of the two dimers, to any other subunit was 2 in whole IAP molecule. The "two-step" treatment of IAP with 5 M urea followed by 8 M urea was essential for preparation of the five constituent subunits each in good homogeneity and yield, since direct exposure of the native IAP to 8 M urea caused a very poor yield of the S-2 subunit for unknown reasons.

Third, mixing these purified subunits, S-1 to S-5, together at the same molar ratio as that found in the native IAP produced a single major protein band that migrated, upon electrophoresis, at the rate corresponding to IAP purified from the culture medium of *B. pertussis*. It was not reproduced if any of the subunits was omitted. This reconstituted protein displayed the IAP activity, though its potency was somewhat less than native IAP probably because of slight denaturation and liable formation of aggregates during isolation of subunits and their recombination (see below). The molecular weight calculated by summing up those of the constituent subunits was 107 000, which is not at variance with the value of 117 000 obtained by the sedimentation equilibrium method and 103 000 reported by Arai & Sato (1976) for their pertussis protein.

The subunit structure of IAP thus assessed conforms to the "A–B" model which was proposed for several bacterial toxins having specific ability to pass through a cell's plasma membrane and to catalyze ADP-ribosylation of its cellular protein (Gill, 1978). In these toxins are included diphtheria toxin (Honjo et al., 1968), cholera toxin (Moss & Vaughan, 1979), *Pseudomonas* exotoxin (Iglewski & Kabat, 1975), *Escherichia coli* enterotoxin (Gill & Richardson, 1980), and pertussis toxin, IAP (Katada & Ui, 1982a,b). According to this model, a catalytically active single peptide (an A component) must be readily separated from the residual peptide(s) (a B component) which, by binding to a particular membrane receptor, facilitates the insertion of the A component into the cell. In the present paper, we have adopted the terms an A protomer and a B oligomer for the biggest subunit and the association product to other subunits, respectively; the experimental basis supporting this terminology is as follows.

The IAP molecule was dissociated to their four components, two monomers (the biggest and the smallest subunits) and two

Table III: Subunit Assembly in IAP Molecule

assembly level	components
0: monomers	S-1 (=A protomer); S-2; S-3; S-4; S-5 (=C subunit)
1: dimers	dimer 1 = [S-2] [S-4]; dimer 2 = [S-3] [S-4]
2: pentamer (=B oligomer)	[dimer 1] [C subunit] [dimer 2]
3: hexamer (=native IAP)	[A protomer] [B oligomer]

dimers, in 5 M urea. The smallest subunit (C subunit) was capable of connecting two dimers, thereby producing a pentamer. Thus, IAP is first separable into the biggest subunit and the residual pentamer as actually observed upon haptoglobin affinity chromatography in the presence of 5 M urea (Figure 7). The pentamer should be a B (binding) component because it was a moiety of the IAP molecule that binds the haptoglobin; the interaction of pertussis toxin with this sialoprotein was considered to afford a model system for the toxin binding to particular membrane receptors (Irons & MacLennan, 1978). On the other hand, the biggest subunit should be an A (active) component in the sense that it confers the biological activity in vivo on the whole IAP (Table II). In fact, the A protomer by itself was as effective as the native IAP in the broken-cell preparation of C6 cells (Figure 10), despite the fact that it failed to exhibit biological activity in vivo unless it was combined with the B oligomer (Table II). Probably, the membrane preparation from C6 cells may possess certain enzymic activity that is responsible for releasing the A protomer from the whole toxin molecule; the A protomer did, but IAP did not, hydrolyze NAD to ADP-ribose and nicotinamide in the absence of cellular components (T. Katada, M. Tamura, and M. Ui, unpublished results). The stepwise assembly of IAP protomers and oligomers is briefly summarized in Table III.

The B oligomer is somewhat unstable by itself; it tended to form an insoluble aggregate during storage in 2 M urea, e.g., reflecting in less color intensity of the main band in lane E than of the main bands in other lanes on the electrophoretogram in Figure 6. Occasionally, it gave a broad, rather than sharp, band probably as a result of its disaggregate into constituents upon electrophoresis. This instability of the B oligomer made it nonfeasible to measure its molecular weight by means of the sedimentation equilibrium method. Neither aggregation nor disaggregation took place, however, during the simple preparation of the B oligomer by affinity chromatography (Figure 7) as revealed by the elution profile from a Sephacryl column (Figure 9, inset), although no absolute value was obtainable for its molecular weight by this gel filtration method as discussed above. Further studies are now in progress on the biological role of this oligomer as well as the A protomer and C subunit.

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