Two Types of Sugar-binding Protein in the Labellum of the Fly

Putative Taste Receptor Molecules for Sweetness

MAMIKO OZAKI, TAISAKU AMAKAWA, KOICHI OZAKI, and FUMIO TOKUNAGA

From the Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka 560, Japan; and Department of Sciences for Human Environment and Biology, Faculty of Human Development and Science, Kobe University, Nada, Kobe 657, Japan

ABSTRACT   Flies have taste cells specifically sensitive to sweetness. It has been suggested that the cells possess two types of receptor sites covering the receptive field of sweetness. By affinity electrophoresis with the site-specific inhibitory polysaccharides, two types of sugar-binding protein were isolated from the labellar extract of the blowfly. These proteins showed consistent sugar-binding specificities and affinities with the two types of receptor sites for sweetness, respectively. The dissociation constant of the protein–sugar complex varies 100–400 mM and the molecular weight of one type of the protein is 27,000, while that of the other is 31,000 or 32,000. Both proteins were water insoluble and were also detected in the isolated chemosensilla. Thus they are probably located on the taste receptor membrane, and the proteins are likely to act as the taste receptor molecules for sweetness in the fly.

INTRODUCTION

Tastes of pure substances are empirically categorized into several elementary groups, i.e., saltiness, bitterness, sourness, sweetness, and umami, which is the taste of some amino acids and nucleotides. Roughly speaking, we sense the tastes of natural substances as various mixtures of some of these elementary tastes. Therefore, it is presumed that the transduction mechanism or the receptor cell might be specialized for each elementary taste.

Recent improvements in the electrophysiological techniques have allowed us to investigate the transduction mechanisms in some taste cells. Avenet, Hofmann, and Lindemann (1988) and Tonosaki and Funakoshi (1988) independently reported with frog and mouse that cyclic nucleotide acts as an excitatory intracellular messenger. The molecular biological study of McLaughlin, McKinnon, and Margolskee (1992) showed that gustducin, a novel G protein closely related to transducin, is expressed

Address correspondence to Dr. Mamiko Ozaki, Department of Biology, Faculty of Science, Osaka University, Toyonaka, Osaka 560, Japan.

in the taste buds of rat. Since the pioneering work of Dastoli and Price (1966) to purify a sweet-sensitive protein, repeated attempts have been made to identify the taste receptor protein in both vertebrates and invertebrates, but taste receptor proteins have not yet been definitively identified. In the fly, Hansen (1969) hypothesized that an α-glucosidase is identical to the sugar receptor protein, and the following studies were concentrated on this enzyme (Hansen and Wieczorek, 1981). However, some inconsistencies with this hypothesis have been reported for the effects of the inhibitors on the enzyme (Nakashima, Enomoto, Kijima, and Morita, 1982). Thus, identifying the molecules triggering the transduction mechanisms in taste cells is still a big problem.

Inconveniently, in vertebrate taste systems, characterization, identification, and classification of the individual receptor cells within a taste bud have not been completely established. The chemosensillum of the fly is a precisely studied taste organ showing simpler structure than the taste bud of vertebrates. Basically, a single chemosensillum possesses four functionally specialized taste cells. One of them, called the sugar receptor cell, is highly sensitive to various sugars, and is suggested to bear at least two different types of sugar receptor sites (Shimada, Shiraishi, Kijima, and Morita, 1974). These receptor sites, each of which has been electrophysiologically characterized in the stimulus selectivity, are called the furanose site (F site) and the pyranose site (P site), respectively. Previously, one of us detected a putative P site protein from the labellar extract of the blowfly by a novel affinity electrophoresis (Ozaki, 1988). At that time, she did not further investigate the protein, but her method can be used to quantitatively evaluate the interaction between the receptor site protein and the stimulus sugar. This time, we detected the F site protein candidate by her method and compared the properties of the F and P site protein candidates, considering the electrophysiological data on each corresponding site.

MATERIALS AND METHODS

Fly

We used adult blowflies, *Phormia regina*, 5–7 d after emergence, reared in our laboratory at 25°C and fed with 0.1 M sucrose.

Electrophysiology

Responses from the chemosensillum of L-type (largest type) blowflies were recorded by the tip-recording method (Hodgson, Lettvin, and Roeder, 1955), by which the impulses from a single sugar receptor cell can be easily recorded. The magnitude of response was defined as the number of impulses during 0.2 s, starting at 0.15 s after the beginning of stimulation. The duration of each stimulation was ~0.5 s and the interval between stimuli was >3 min, which is long enough to diminish the adaptation effect of the last stimulation.

The stimulus sugar solutions were prepared by serial fourfold dilutions with 10 mM NaCl carried out six times for each sugar and starting with 2 M monosaccharides and 1 M disaccharides in 10 mM NaCl, respectively. To construct a pair of concentration–response curves (one as the test for the inhibitory effect of polysaccharide and the other as its control), responses to the serial concentrations of a sugar with or without an arbitrary concentration of starch or levan were recorded from the same cell.

In a series of experiments, data were obtained from a single batch of flies; for some unknown

reason, the maximum response or the midpoint concentration sometimes differs between batches. One can find such differences in K_b for the stimulation with D-fructose between Hara's data (1983) and ours in Table I.

The ambient temperature was 20–25°C and the relative humidity was kept above 70% in the course of the experiments.

TABLE I

Inhibition Effects of Polysaccharides and Estimates of the Inhibition Constants

Polysaccharide (concentration)	Sugar (binding site)	$R_m \pm$ SD	$K_b \pm$ SD	No. of tests	$K_i \pm$ SD
%		mM	mM		%
Levan					
(0)	D-Fructose	17.0 ± 1.8	28.3 ± 1.9	10	—
(1)	(F)	15.2 ± 0.4	62.0 ± 2.5	5	0.82 ± 0.10
(2)		15.2 ± 0.4	105.0 ± 7.1	5	0.85 ± 0.07
(0)	D-Fucose	20.8 ± 1.2	18.3 ± 2.6	6	—
(1)	(F)	21.2 ± 1.5	40.2 ± 4.4	6	0.84 ± 0.10
(2)		21.3 ± 1.6	62.0 ± 7.5	6	0.82 ± 0.05
(0)	D-Glucose	21.6 ± 1.1	79.6 ± 11.1	5	—
(2)	(P)	20.4 ± 2.1	79.6 ± 9.9	5	∞
(0)	L-Fucose	22.8 ± 1.5	54.5 ± 6.2	4	—
(2)	(P)	22.3 ± 1.3	54.5 ± 6.2	4	∞
(0)	Sucrose	22.0 ± 2.6	19.3 ± 2.5	6	—
(2)	(P)	21.3 ± 2.6	20.2 ± 3.5	6	∞
Starch					
(0)*	D-Fructose	17.7 ± 1.7	50.0 ± 11.0	9	—
(2)*	(F)	17.6 ± 2.6	55.0 ± 19.0	9	∞
(0)	D-Fucose	22.6 ± 3.4	25.0 ± 4.1	5	—
(2)	(F)	22.6 ± 3.4	25.6 ± 6.6	5	∞
(0)*	D-Glucose	22.0 ± 0.8	83.0 ± 27.0	8	—
(1)*	(P)	23.3 ± 0.5	228.0 ± 66.0	8	0.68 ± 0.04
(2)*		29.1 ± 1.6	335.0 ± 53.0	8	0.68 ± 0.25
(0)	L-Fucose	23.0 ± 1.4	54.5 ± 3.4	4	—
(1)	(P)	22.8 ± 1.7	112.5 ± 9.6	4	0.73 ± 0.04
(2)		23.0 ± 0.0	175.0 ± 12.9	4	0.66 ± 0.02
(0)*	Sucrose	25.9 ± 1.6	15.0 ± 3.0	10	—
(1)*	(P)	26.6 ± 1.8	43.0 ± 11.0	10	0.64 ± 0.07
(2)*		24.2 ± 2.6	72.0 ± 13.0	10	0.58 ± 0.02

*Data cited from Hara (1983).

Sample Preparation

Living flies were anesthetized by cooling on ice, and the labella were cut at the distal end of the proboscis. The collected labella were frozen and homogenized with a small volume of liquid nitrogen in a hand mortar. The homogenate was suspended in the sample buffer (4.75 mM sodium barbiturate-HCl, 2% Triton X-100, and 10% glycerol, pH 6.8), incubated at 4°C for 1 h, and centrifuged at 3,000 rpm at 4°C for 10 min. At one time, 10–30 μl of the supernatant (50–100 labella) was applied to the electrophoresis.

When the isolated chemosensilla were used as the sample, proboscises were first collected in the 2-ml plastic sampling tube (200–500 proboscises per tube), frozen by dipping the tube in liquid nitrogen, and immediately vortexed for 5 s. This freeze-vortex was repeated three times

and the contents were discarded by turning the tube upside down. However, the isolated chemosensilla were still kept on the inner wall of the tube by the static force, so that they were then scraped with a small piece of the printing paper. The homogenate of the isolated chemosensilla was made in a small glass homogenizer instead of a hand mortar.

Electrophoresis

Since many different kinds of proteins are present in the blowfly labellum, it was difficult to detect a minor protein such as the sugar receptor protein in one-dimensional electrophoresis. Therefore, we adopted a two-dimensional polyacrylamide gel electrophoresis, in which the first and the second dimensional electrophoretic systems are the same but an affinity ligand was uniformly mixed in the running gel of the first dimension only. As the affinity ligands, we used the polysaccharides that interact with the sugar receptor sites as the competitive inhibitors. Since the polysaccharides are large molecules without electric charges, they are useful as the immobile affinity ligand when simply mixed in the polyacrylamide gel. The proteins that interact with the polysaccharide retard in the first dimensional run but recover their migration speeds in the second dimensional run. Every other protein that does not interact with the polysaccharide migrates at the same speed in both the first and second dimensional runs. As a result, the proteins that interact with the polysaccharide are detected as spots separate from a big diagonal stain of other proteins. We call this two-dimensional electrophoresis the diagonal method. The gel system was basically the same as the system of Davis (1964) and Ornstein (1964) and has been cited in detail in Ozaki (1988). The stacking and running gels contained Triton X-100 (2% at the final concentration) and the barbiturate buffers were used instead of Tris buffers, which inhibit the sugar responses. Proteins were detected by the silver staining method of Oakley, Kirsh, and Morris (1980).
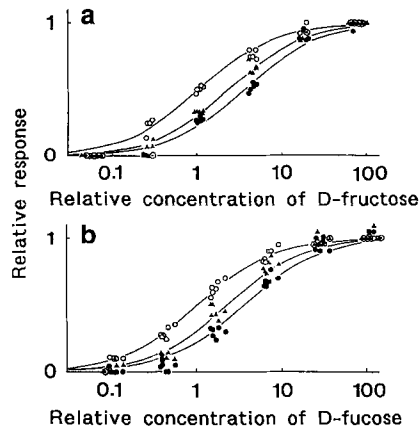
Chemicals

We used soluble starch purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and levan (No. L-4884) purchased from Sigma Chemical Co. (St. Louis, MO). Recently, Sigma Chemical Co. has switched the production of levan from *Aerobacter levanicum* (No. L-4884) to *Erwinia herbicola* (No. L-8647), but we did not use the levan from *Erwinia* (No. L-8647).

RESULTS

Selective Inhibition of the Sugar Receptor Sites by Polysaccharides

The inhibitory effects of several polysaccharides were once investigated on the taste response of the labellar sugar receptor cell of the blowfly (Hara, 1983 [paper published under M. Ozaki's maiden name]). Among the examined polysaccharides, starch, which consists of glucopyranose residues, competitively inhibits the P site and was used as an affinity ligand for the P site protein (Ozaki, 1988). However, any adequate F site–specific competitive inhibitors usable as the affinity ligand were not found. Thus, we again tried the electrophysiological inhibition experiment with polysaccharides. As the stimulus sugars, we used D-fructose and D-glucose because they are the representative furanose and pyranose, respectively. We also examined D- and L-fucoses because these optical isomers stimulate the F and P sites, respectively (Shimada et al., 1974), and sucrose because it is one of the most familiar sweet substances for us and a representative P site–stimulative disaccharide for the fly.

As shown in Fig. 1, when levan, a polysaccharide consisting of fructofuranose
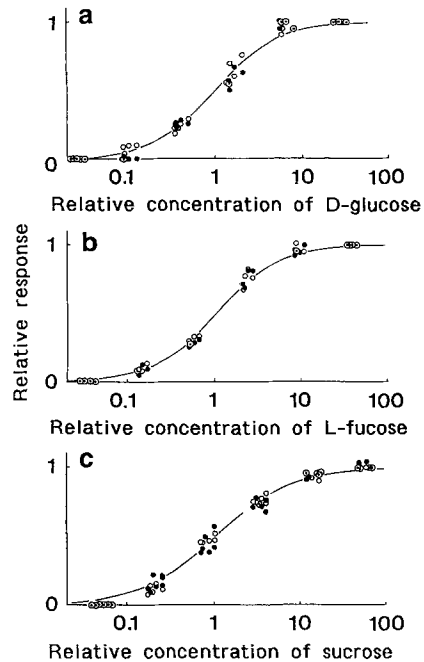
FIGURE 1. Effect of levan on the concentration–response curves for the F site stimulative sugars in the blowfly sugar receptor cell. The response was recorded to D-fructose (*a*) and D-fucose (*b*) in the absence or presence of levan. In Figs. 1 and 2, the magnitude of the response being normalized to the maximum response in the absence of levan (relative response) was plotted against the logarithmic scale of the normalized sugar concentration to the midpoint concentration in the absence of levan (relative concentration). The continuous line indicating the theoretical equation (Eq. 5 in text) was fitted by eye to the response to pure sugar solutions (○) with $n' = 1$ in *a* and *b*, and was simply shifted to the right to fit the response in the presence of 1 (▲) or 2% (●) levan.

residues, was mixed in the stimulus solution of D-fructose (Fig. 1 *a*) or D-fucose (Fig. 1 *b*), the concentration–response curve simply shifted to the right. When levan was mixed in the stimulus solution of D-glucose (Fig. 1 *a*), L-fucose (Fig. 1 *b*), or sucrose (Fig. 1 *c*), however, the concentration–response relationship did not change (Fig. 2). These results suggested that levan, a fructofuranose residue of which can fit the F site, competed with D-fructose or D-fucose, but could not interact with the P site.

FIGURE 2. Effect of levan on the concentration–response curves for the P site stimulative sugars in the blowfly sugar receptor cell. The response was recorded to D-glucose (*a*), L-fucose (*b*), or sucrose (*c*) in the absence or presence of levan. The continuous line was fitted with $n' = 1.2$ in *a* and *b* and $n' = 1$ in *c*. The symbols are the same as in Fig. 1.

Table I summarizes the site-specific inhibitory effects of levan as well as those of starch. Even when a given polysaccharide was effective, the maximum response, R_m, did not significantly change. However, the midpoint concentration, K_b, which is the sugar concentration giving rise to the half-maximum response, was increased.

Based on the increases in the midpoint concentrations (from K_b to K'_b), we can calculate the inhibition constants of polysaccharides, according to the following equation (Rang, 1971):

$$K_i^n = [I]^n/([S]'^{n'}/[S]^{n'} - 1) \tag{1}$$

where K_i is the inhibition constant of a competitive inhibitor if the concentration [S] of a pure solution of a sugar produces the same magnitude of response as the concentration of [S]' in the presence of a concentration [I] of the inhibitor, and n and n' are the Hill coefficients for the inhibitor and the stimulant, respectively. The value of n' can be obtained as the slope of the concentration–response curve at [S] = K_b, and n is 1 because of the large molecular form of the inhibitor.

Assuming the simple case that one molecule of D-fructose or D-fucose binds to one molecule of the F site ($n' = 1$) and levan also makes a 1:1 complex with the F site ($n = 1$), the inhibition constant of levan for the F site is calculated to be 0.82–0.85% (with [S] = K_b and [S]' = K'_b). This value was independent of the kind of stimulus sugar or the concentration of levan. The inhibition constant of starch for the P site was once estimated to be 0.6–0.7% from the inhibition experiment on the response to D-glucose, sucrose, or maltose by Hara (1983; her data are cited in Table I). We further examined the inhibitory effect of starch on the response to D- and L-fucoses and obtained consistent results; i.e., starch did not affect the response to D-fucose but inhibited the response to L-fucose with almost the same inhibition constant ($K_i = 0.72\%$, assuming $n = 1$ and $n' = 1.2$) as the previous estimate. K_i values determined in this way are listed in the right column of the Table I, where ∞ means the calculated value is >20%. The K_i values could not be expressed in molar concentration but in % wt/vol, because both levan and starch are polydisperse.

When Ozaki (1988) reported the P site protein candidate, she noted that the protein was not identical with the α-glucosidase that had been proposed to be a receptor protein (Hansen, 1969). Actually, starch could not affect the binding of sugars to the α-glucosidase. This discounts the possibility that the polysaccharides prevented the sugar–receptor interaction by binding the sugar stimuli and resulted in the concentration–response curve shift as shown in Fig. 1.

Detection of the Sugar-binding Proteins on the Affinity Gel

Fig. 3 shows the gel patterns into which the Triton X-100 extract of labella was separated by the diagonal method (see Materials and Methods). Under the no polysaccharide condition, every protein migrated on a diagonal line (Fig. 3 a). When 1 (Fig. 3 b) or 1.5% levan (Fig. 3 c) was added to the running gel for the first run, a spot of the F site protein candidate was separated from the diagonal stain, depending on the concentration of levan. The similar electrophoretic pattern is seen in Fig. 3 d, but an isolated spot indicates that the P site candidate protein was separated by the diagonal method with 1.5% starch. By superimposing Fig. 3, c and d with each other,
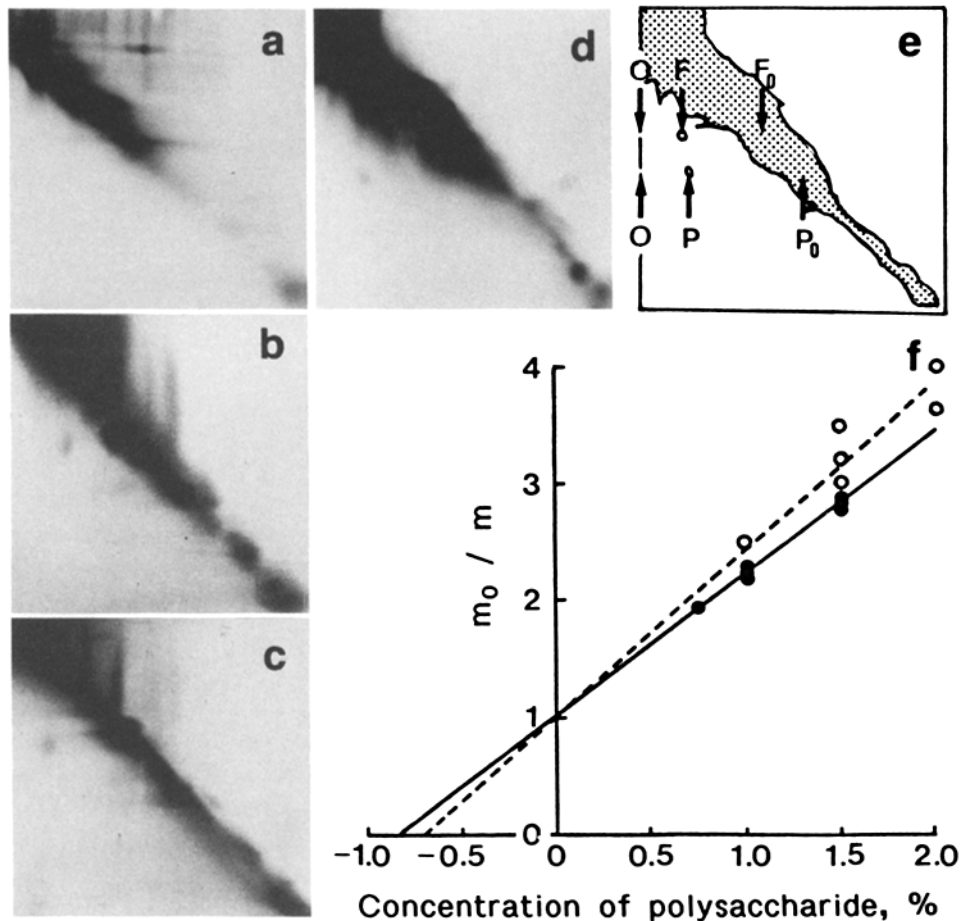
FIGURE 3.   Affinity electrophoretic patterns of the labellar extract by the diagonal method; (*a*) 0%; (*b*) 1%; (*c*) 1.5% levan, and (*d*) 1.5% starch in the first dimension. The origin of the two-dimmensional electrophoresis is the upper left corner in each gel of Figs. 3, 4, and 6. Proteins migrated toward the right and bottom in the one- and two-dimensional electrophoreses, respectively. (*e*) The superimposed drawing of *c* and *d*; F and P, the isolated spots for the F and P proteins, respectively; F_0 and P_0, the positions to which the F and P proteins would have migrated in the absence of either levan or starch. F_0 and P_0 are determined as the crossing points of the horizontal extensions of lines OF and OP with the diagonal line defined by the protein stain (*dotted area*). When the protein stain appeared smeared in the central portion, the diagonal line was defined as the straight line from the origin of the electrophoresis to some distinguishable spots near the leading front of protein migration. (*f*) Determination of the dissociation constants of the F protein–levan complex by plotting the reciprocals of the relative mobilities of the proteins, m_0/m, against the concentrations of levan, [I] (●). The mobilities, *m* and m_0, are proportional to the distances OF and OF_0, respectively. A similar plot on the P protein–starch complex can also be seen. A broken line is drawn based on Ozaki's data (1988). The data we obtained in this experiment (○) were well fit to the broken line.

the migration positions of the F (F_0) and P site candidate proteins in the absence of polysaccharide (P_0) are presumed to be different from each other (Fig. 3 e).

For a simple presentation, we would like to tentatively call these receptor protein candidates "F protein" and "P protein," respectively.

Both levan and starch are large molecules with no electric charge, which, when complexed with a protein in the gel, greatly retard the mobility of the protein during electrophoresis. Therefore, the protein–levan or protein–starch interaction can be expressed by the following equation (Takeo and Nakamura, 1972):

$$m_0/m = 1 + [I]/K_i' \qquad (2)$$

where m_0 and m are the mobilities in the absence and presence of levan or starch of concentration [I]. K_i' is the dissociation constant of the protein–levan or protein–starch complex. The ratio of the mobilities m_0/m was easily determined from the electrophoretic patterns of the diagonal method (Fig. 3 e). Thus, the plot of m_0/m against [I] yielded a straight line with an intercept at $-K_i'$ (Fig. 3 f). The K_i' of the F protein–levan complex was estimated to be 0.83%. It is identical to an electrophysiological estimate of the inhibition constant of levan for the F site, which is defined as the dissociation constant of the F site–levan complex (Table I). The K_i' of the P protein–starch complex was already reported by Ozaki (1988) to be 0.6–0.7% and almost the same as the result in our reexamination. This was also identical with the electrophysiological estimate of the inhibition constant of starch to the P site.

Binding Abilities of the Proteins to Sugars

The stimulative sugar is generally small, so that when a protein makes a complex with the sugar, the mobility of the complex in the gel is expected to be nearly equal to that of the free protein. In the presence of levan plus D-fructose in the first run of the electrophoresis, a spot was detected closer to the diagonal stain (Fig. 4 b) than in the presence of levan alone (Fig. 4 a). This indicated that D-fructose competes with levan for the F protein, because the F protein–D-fructose complex can migrate faster than the F protein–levan complex. When D-glucose was used instead of D-fructose, a spot was detected at almost the same position (Fig. 4 c) as the F protein spot in the presence of levan alone (Fig. 4 a). The F protein would not form a complex with D-glucose, so it migrated as if the gel included only levan in the first run.

If the mobility, m, of a protein in the presence of levan alone becomes m' in the presence of levan (concentration, [I]) plus a stimulus sugar (concentration, [S]), the 1:1 interaction between protein and sugar is given by the following equation (Takeo and Nakamura, 1978):

$$m'/(m_0 - m') = K_i'(1 + [S]/K_d')/[I] \qquad (3)$$

where K_d' is the dissociation constant of the protein–sugar complex. Thus, the plot of $m'/(m_0 - m')$ against [S] gives a straight line intercepting the [S] axis at $-K_d'$. Fig. 4 d shows such a plot on the F protein–D-fructose interaction, suggesting a 1:1 complex formation with $K_d' = 341$ mM. If m' is equal to m as shown in Fig. 4 c, Eqs. 2 and 3 lead K_d' to be infinitely large and Eq. 3 becomes $m'/(m_0 - m') = K_i'/[I]$. Thus, the plot of $m'/(m_0 - m')$ against [S], showing a parallel line to the [S] axis (Fig. 4 e), implicates no interaction between the F protein and D-glucose.
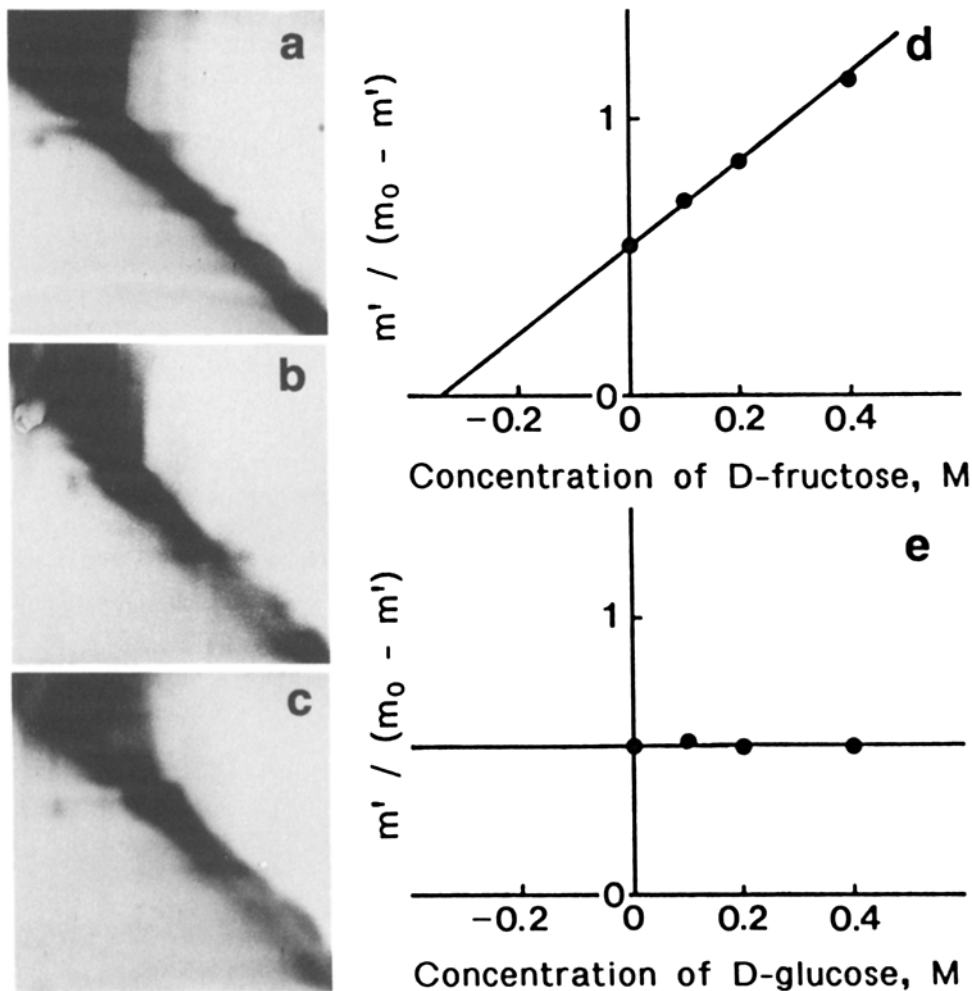
FIGURE 4. Interactions of the F protein with D-fructose and D-glucose. Affinity electrophoretic patterns of the labellar extract by the diagonal method. (*a*) 1.5% levan plus no sugar; (*b*) 1.5% levan plus 0.1 M D-fructose; (*c*) 1.5% levan plus 0.1 M D-glucose in the first dimension; (*d*) determination of the dissociation constants of the F protein–D-fructose complex by plotting $m'/(m_0 - m')$ against the sugar concentration, [S]; (*e*) determination of the dissociation constant of the F protein–D-glucose complex.

The dissociation constants of the receptor protein candidate–stimulus sugar complexes, K'_d, estimated by affinity electrophoresis, and the electrophysiologically determined midpoint concentrations, K_b, of the stimulative sugars for the two receptor sites are listed in Table II. As for the sugars used here, the K'_d varied between 103 and 360 mM. However, a clear parallelism was found between the K'_d and K_b; i.e., $K'_d = 12\ K_b$ on the F protein and $K'_d = 5\ K_b$ on the P protein. Previously, Ozaki (1988) presented $K'_d = 4\ K_b$ on the P protein, but actually there were no big discrepancies between her data and ours.

TABLE II

Comparison of K_b with K'_d for the F and P Proteins

Sugar (binding site)	$K_b \pm SD$ (No. of tests)	F protein		P protein	
		$K'_d \pm SD$ (No. of tests)	K'_d / K_b	$K'_d \pm SD$ (No. of tests)	K'_d / K_b
	mM	mM		mM	
D-Fructose (F)	28.3 ± 1.9 (10)	341.2 ± 13.5 (3)	12.1	∞ (3)	—
D-Fucose (F)	18.3 ± 2.6 (6)	214.7 ± 13.4 (3)	11.7	∞ (3)	—
D-Glucose (P)	79.6 ± 11.1 (5)	∞ (3)	—	360.0 ± 25.0 (4)	4.5
L-Fucose (P)	54.5 ± 3.4 (4)	∞ (3)	—	260.0 ± 18.3 (4)	4.8
Sucrose (P)	19.3 ± 2.5 (6)	∞ (3)	—	103.5 ± 25.3 (4)	5.4

Molecular Weight and Location of the Sugar-binding Proteins

After the affinity electrophoresis by the diagonal method with levan or starch, small gel pieces (5 mm × 5 mm) containing the F and P protein spots, respectively, were cut out and applied to SDS-PAGE for molecular weight determination. The F protein was identified as a single spot of ~27 kD (Fig. 5, lane a). However, the P protein,
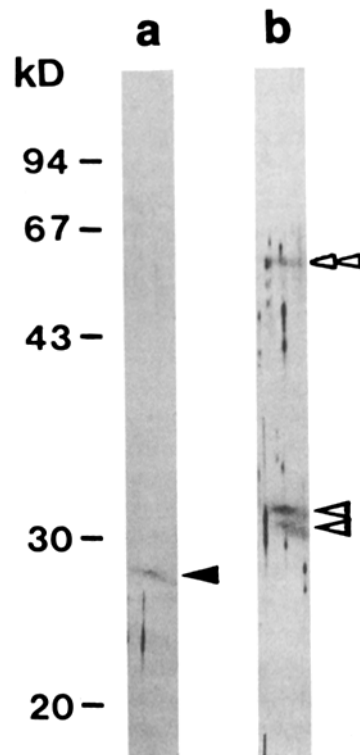

FIGURE 5. Determination of the molecular weights of the F (a) and P proteins (b) by the SDS-polyacrylamide electrophoresis.

which apparently forms a single spot by the diagonal method, was found as twin spots of ~31 and 32 kD (Fig. 5, lane *b*). Another spot of ~63 kD is also seen in lane *b* (double open arrowhead). It might be a dimer of the P protein. We reexamined the same SDS-PAGE under the β-mercaptoethanol-free condition but the results were the same as these.
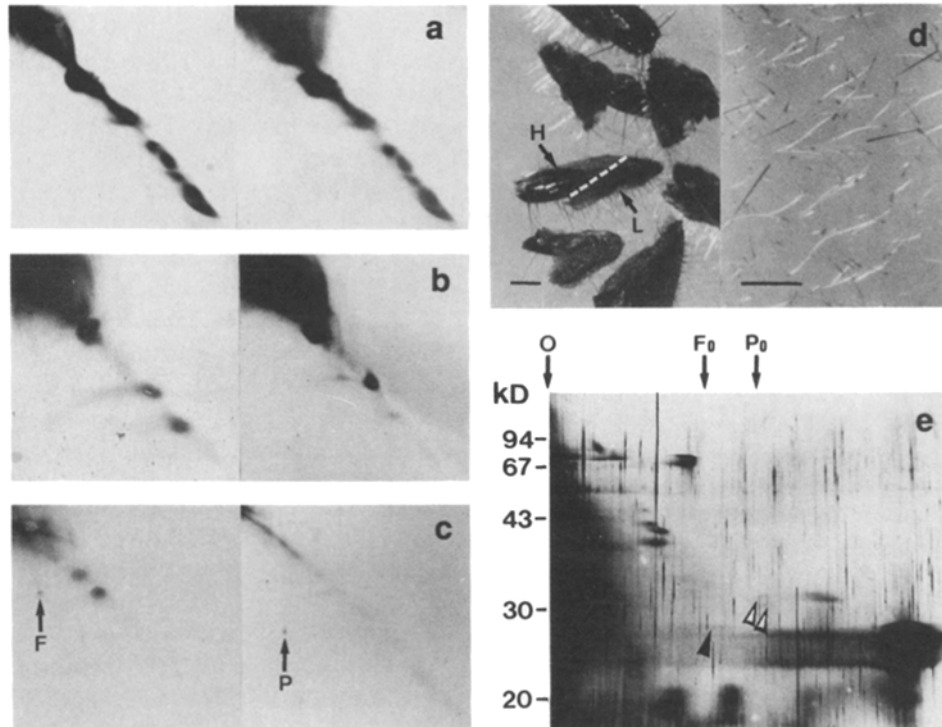

FIGURE 6. Detection of the F and P proteins in parts of the proboscis. The electrophoretic patterns by the diagonal method with 1.5% levan (*left*) or starch (*right*). (*a*) Water-soluble extract of the labella (from 75 flies); (*b*) Triton X-100 extract of the haustellum (from 75 flies); (*c*) Triton X-100 extract of the isolated chemosensilla (from 700 flies); (*d*) proboscises including labella (L) and haustella (H) (*left*), and isolated chemosensilla from the proboscises (*right*) (bars indicate 500 μm); (*e*) native-SDS two-dimensional electrophoretic pattern of the Triton X-100 extract of isolated sensilla (from 1,300 flies). The one-dimensional electrophoresis was the same as that in the diagonal method, but without polysaccharide. F_0 and P_0 were estimated from the affinity electrophoresis by the diagonal method with levan or starch.

The taste cell of the fly is a primary sensory cell extending a sensory process to the sensillum and an axon to the brain through the haustellum. Therefore, the labellum (L in Fig. 6 *d, left*), having a number of sensilla, includes the sensory processes, cell bodies, and short pieces of axons (labial nerve) of the taste cells. Although we ordinarily used Triton X-100 extract of labella in these experiments, the water-soluble components of the labellar extract could be easily examined by the diagonal method. The labellar homogenate was incubated in the same sample buffer without

Triton X-100 and the supernatant was applied to the electrophoresis. Triton X-100 was also removed from the gels in both dimensions, so that only the water-soluble components could come into the gel. Such a detergent-free electrophoresis did not result in detection of either the F (Fig. 6 *a*, *left*) or the P protein (Fig. 6 *a*, *right*). Thus, the proteins are likely to be membrane bound rather than water soluble.

The haustellum (*H* in Fig. 6 *d*, *left*), the proximal part next to the labellum in a proboscis, includes pieces of the axons of the taste cells. When we examined the Triton X-100 extract of haustellum, neither the F (Fig. 6 *b*, *left*) nor the P protein (Fig. 6 *b*, *right*) could be detected. Thus, these proteins may be poorly distributed in the axons.

We also succeeded in isolating the chemosensilla by vortexing the frozen proboscises (Fig. 6 *d*, *right*). Fig. 6 *c* shows the electrophoretic patterns of the Triton X-100 extract of the isolated sensilla by the diagonal method with 1.5% levan or starch. The tiny spots for the F (*left*) and P proteins (*right*) can be seen at the expected positions (cf. Fig. 2, *a* and *c*). Since each chemosensillum contains the sensory processes of four taste cells but no other cellular components, these proteins may locate in at least one of the sensory processes. Fig. 6 *e* shows a native-SDS two-dimensional polyacrylamide gel electrophoretic pattern of the isolated chemosensillar extract. The twin spots of 31 and 32 kD for the P protein (open arrowheads) are easily detected. We also indicate a spot of 27 kD as the most likely F protein spot (closed arrowhead).

The sensory processes within the chemosensillum possess few intracellular membrane structures. Therefore, our results suggested that the F and P proteins locate on the surface membrane of the sensory processes, i.e., the receptive region of the taste cell.

DISCUSSION

Affinity Electrophoresis

We determined that the dissociation constants of the receptor candidate–sugar complexes (K'_d) were > 100 mM. The ordinary biochemical techniques, for instance, affinity chromatographies or binding assays with the labeled stimulants, are not applicable to isolate the receptor protein because of such a weak receptor protein–stimulant interaction. To solve this problem, one of us introduced an affinity electrophoresis with polysaccharide and first detected the P protein (Ozaki, 1988). Here we used levan as the affinity ligand for the F protein, expecting that the levan molecule would be large enough to be immobilized in the polyacrylamide gel like starch. Fig. 3 shows a linear relationship between m_0/m and [I] as demanded by Eq. 1. This suggested that the F protein, when it made a 1:1 complex with levan, was indeed immobile in the polyacrylamide gel during electrophoresis.

The second problem was the diversity of the taste receptor proteins and the small content of each of them. The content of the P site protein has been calculated from the electrophysiological data to be 10^{-13}–10^{-10} g per fly (Hansen and Wieczorek, 1981). However, even this small amount of protein can be detected by the highly sensitive silver staining on the polyacrylamide gel if 50–100 labella are used. Actually, the P protein content presumed by its staining limit was consistent of the electrophys-

iological estimate (Ozaki, 1988) and the content of the F protein was not very different from that of the P protein.

Dissociation Constant and Midpoint Concentration

The taste receptor–stimulant interaction was first theoretically analyzed by Beidler (1954). He introduced the electrophysiological constant of the midpoint concentration as the dissociation constant of the receptor–stimulant complex itself in his article. However, one can find that the dissociation constants of the F protein–sugar or the P protein–sugar complexes, K'_d, are larger than the corresponding electrophysiological midpoint concentrations, K_b. Assuming that the ion channel opening on the receptor membrane is triggered by the stimulus sugar molecule, making 1:1 complex with the receptor site, an equivalent circuit introduced by Morita (1969, 1992) leads to the following equation on the relative response:

$$R/R_m = 1/\{1 + K_d/(1 + sg/G)[S]\} \tag{4}$$

where the magnitude of response, R, is defined as the number of impulses generated 0.15–0.35 s after the beginning of stimulation. The impulse frequency during this period is proportional to the receptor potential (during the initial period before that, a burst of impulse discharge that was not linearly controlled by the receptor potential was observed), and hardly affected by adaptation (Ozaki and Amakawa, 1992). R_m can be obtained as the magnitude of the response to 1 M disaccharide or 2 M monosaccharide (Morita, 1969), K_d is the dissociation constant of the receptor site–sugar complex, s is the total number of ion channels operated by the stimulant-bound receptor, g is the conductance per channel, and G is the conductance across the receptor membrane in the resting state. On the other hand, the concentration–response relationship obtained by plotting the relative response, R/R_m, against [S] in the logarithmic scale is well fitted to the following equation (continuous lines in Figs. 1 and 2):

$$R/R_m = 1/(1 + K_b/[S]) \tag{5}$$

Comparing Eq. 5 with Eq. 4,

$$K_d/K_b = 1 + sg/G \tag{6}$$

Thus, the dissociation constant of the receptor site–sugar complex, K_d, must be larger than the electrophysiological midpoint concentration, K_b, because $sg/G > 0$.

Our results showed that $1 + sg/G = 12$ on the F site and 5 on the P site. If the F and P proteins actually construct the F and P sites, respectively, and K'_d directly reflects on K_d, this indicates that the intracellular transduction process for sweetness in the fly is not single but is divided into at least two routes. They are via F and P sites, and are characterized by the different values of s and/or g, respectively.

Twin Spots of the P Protein and Subsite Hypothesis for the P Site

The Hill coefficient of the concentration–response relationships is 1.0 when monosaccharide stimulates the F site and disaccharide stimulates the P site. When monosaccharide stimulates the P site, however, the Hill coefficient is between 1.0 and 2.0. This suggests that the P site makes a 1:1 complex with disaccharides like F

site–monosaccharide complexes, but that the P site can make 1:2 complexes with monosaccharides. Morita and Shiraishi (1968) proposed a subsite hypothesis to interpret the sugar-binding manner of the P site; i.e., the P site consists of two subsites, each of which can be fit by a monosaccharide, and a cooperativity is shown between the subsites. They also imagined that each moiety of a disaccharide fits each subsite that adjoins it, so that the disaccharide fits the P site in a 1:1 manner. This subsite hypothesis may refer to the subunit structure of the receptor protein of the P site.

The P protein appearing as a single spot on the native gel separated into twin spots of 31 and 32 kD on the SDS-polyacrylamide gel. Since there are several different shapes of chemosensilla on the labellum, some of them might have a different type of P protein than others. Otherwise, the twin spots of the P protein may realize the hypothetical subunits based on the subsite hypothesis. In the native gel of the diagonal method, the subunits could migrate together as an intact P protein molecule. A 63-kD spot is seen in Fig. 5, lane *b* (double open arrowhead) in addition to the twin spots of 31 and 32 kD (single open arrowheads). This might be the P protein remaining in the intact form.

Taste Recognition

Considering the essential structures of stimulative sugars, Ninomiya and Shimada (1976) presented the structure models of the F and P sites, in which a few hydrogen bonds can be formed between the receptor site and sugars. According to our calculation based on the dissociation constant of the F or the P protein–sugar complex, the free energy change for the complex formation was estimated to be −1.3 to −0.5 kcal. This energy change corresponds to the partial affinity force due to one or two hydrogen bonds when the glucoamylase forms a complex with α-D-glucose (Hiromi, Kawai, Suetsugu, Nitta, Hosotani, Nagao, Nakajima, and Ohno, 1973). Such a weak receptor–stimulant interaction occurs so that the animal can readily recognize the new stimulant by washing the surface of the receptor membrane with fluid (mucus or saliva in vertebrates, sensillar lymph in the fly).

Bruck and Axel (1991) mentioned in their paper on the huge family of the odorant receptor protein cDNA that olfactory perception probably uses an extremely large number of receptor types, each capable of recognizing a small number of odorous ligands. In the taste receptor, however, the individual receptor protein may have wider stimulus binding ability than the odorant receptor protein. The taste receptor protein obtains the wide stimulus binding ability at the expense of either the fine structural resolution of each stimulant or the high sensitivity to a particular stimulant. It is a completely different point from the internal chemoreceptor molecules like hormone or neurotransmitter receptors.

The taste system of vertebrates should have some differences from that of the fly, but a limited number of receptor types (less than the odorant receptor) might be enough to be responsible for each elementary taste we can recognize. Nevertheless, if the animal can recognize fairly delicate changes of taste, there may be factors other than diversity in the receptor molecule, e.g., modification of or interaction between the receptor molecules, combination of the exciting taste cells, or processing in the central nervous system.

REFERENCES

Avenet, P., F. Hofmann, and B. Lindemann. 1988. Transduction in taste receptor cells requires cAMP-dependent protein kinase. *Nature.* 331:351–354.

Beidler, L. M. 1954. A theory of taste stimulation. *Journal of General Physiology.* 38:133–139.

Bruck, L., and R. Axel. 1991. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell.* 65:175–187.

Dastoli, F. R., and S. Price. 1966. Sweet-sensitive protein from bovine taste buds: isolation and assay. *Science.* 154:905–907.

Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences.* 121:404–427.

Hansen, K. 1969. The mechanism of insect sugar reception: a biochemical investigation. *In* Olfaction and Taste. Vol. III. C. Pfaffmann, editor. The Rockefeller University Press, New York. 382–391.

Hansen, K., and H. Wieczorek. 1981. Biochemical aspects of sugar reception in insect. *In* Biochemistry of Taste and Olfaction. M. Kare, editor. Academic Press, New York. 139–162.

Hara, M. 1983. Competition of polysaccharides with sugar for the pyranose and the furanose sites in the labellar sugar receptor cell of the blowfly, Phormia regina. *Journal of Insect Physiology.* 29:113–118.

Hiromi, K., M. Kawai, N. Suetsugu, Y. Nitta, T. Hosotani, A. Nagao, T. Nakajima, and S. Ohno. 1973. Kinetic studies on glucoamylase. VI. Inhibition of substrate analogues. *Journal of Biochemistry.* 74:935–943.

Hodgson, E. S., J. Y. Lettvin, and K. D. Roeder. 1955. Physiology of a primary chemoreceptor unit. *Science.* 122:417–418.

McLaughlin, S. K., P. J. McKinnon, and R. F. Margolskee. 1992. Gustducin is a taste-cell-specific G protein closely related to the transducins. *Nature.* 357:563–569.

Morita, H. 1969. Electrical signals of taste receptor activity. *In* Olfaction and Taste. Vol. III. C. Pfaffmann, editor. The Rockefeller University Press, New York. 370–381.

Morita, H. 1992. Transduction process and impulse initiation in insect contact chemoreceptor. *Zoological Science.* 9:1–16.

Morita, H., and A. Shiraishi. 1968. Stimulation of the labellar sugar receptor of the fleshfly by mono- and disaccharides. *Journal of General Physiology.* 52:559–583.

Nakashima, M., K. Enomoto, H. Kijima, and H. Morita. 1982. Discrepancy between the affinities of certain inhibitors for the membrane-bound α-glucosidases and those for the sugar receptor of the flies. *Journal of Insect Physiology.* 12:579–585.

Ninomiya, M., and I. Shimada. 1976. Stereospecificity for oligosaccharides of two receptor sites in a labellar sugar receptor of the fleshfly. *Journal of Insect Physiology.* 22:483–487.

Oakley, B. R., D. R. Kirsh, and N. R. Morris. 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Analytical Biochemistry.* 105:361–363.

Ornstein, L. 1964. Disc electrophoresis. I. Background and theory. *Annals of the New York Academy of Sciences.* 121:321–349.

Ozaki, M. 1988. A possible sugar receptor protein found in the labellum of the blowfly, *Phormia regina. Zoological Science.* 5:281–290.

Ozaki, M., and T. Amakawa. 1992. Adaptation-promoting effects of IP_3, Ca^{2+}, and phorbol ester on the sugar taste receptor cell of the blowfly, *Phormia regina. Journal of General Physiology.* 100:867–879.

Rang, H. P. 1971. Drug receptors and their function. *Nature.* 231:91–96.

Shimada, I., A. Shiraishi, H. Kijima, and H. Morita. 1974. Separation of two receptor sites in a single labellar sugar receptor of the fleshfly by treatment with p-chlormercuribenzoate. *Journal of Insect Physiology.* 20:605–621.

Takeo, K., and S. Nakamura. 1972. Dissociation constants of glucan phosphorylase of rabbit tissues studied by polyacrylamide gel disc electrophoresis. *Archives of Biochemistry and Biophysics.* 153:1–7.

Takeo, K., and S. Nakamura. 1978. Dissociation constants of glucan phosphorylases to oligo- and polyglucans studied by the affinity electrophoresis. *In* Affinity Chromatography. Pergamon Press, Oxford and New York. 67–70.

Tonosaki, K., and M. Funakoshi. 1988. Cyclic nucleotides may mediate taste transduction. *Nature.* 331:354–356.