Interaction of β-Lactoglobulin and Cytochrome *c*: Complex Formation and Iron Reduction[1]

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β-Lactoglobulin forms a soluble complex with cytochrome *c* in mildly alkaline solutions of low ionic strength. Sedimentation velocity experiments suggest that the complex (maximum s_{20} = 3.7) consists of one cytochrome *c* molecule per β-lactoglobulin monomer unit. At pH 8 or higher, the presence of β-lactoglobulin causes reduction of ferri- to ferrocytochrome *c*. The initial rate of reduction at a single temperature depends primarily on the concentration of β-lactoglobulin, although the final percentage ferrocytochrome *c* obtained is constant at molar ratios of three or more β-lactoglobulin monomers to one cytochrome *c* molecule. The temperature dependence of the initial rate of iron reduction resembles that for alkaline denaturation of β-lactoglobulin. The displacement of *N*-dansylaziridine, a sulfhydryl specific dye, from bovine β-lactoglobulin during iron reduction, and the formation of nonreducing complexes between the analogous swine protein (no sulfhydryls) and cytochrome *c* suggest that the sulfhydryl group of β-lactoglobulin is the electron donor.

Protein–protein interactions are important in cellular activity. Processes such as the formation of multimeric enzyme complexes, the assembly of structural proteins, and the control of enzymatic activity all involve protein–protein interactions. In addition, studies of interactions between two or more unrelated proteins may help define the properties of the individual proteins or suggest possible mechanisms for interaction of each protein with others in its native milieu. Bovine β-lactoglobulin and horse heart cytochrome *c* have each been carefully studied for many years. However, despite voluminous literature, the biological function of β-lactoglobulin and the precise mechanism by which cytochrome *c* transports electrons are, as yet, not clearly identified.

Previous studies have shown that cytochrome *c* will form complexes with cytochrome oxidase (1), cytochrome peroxidase (2), and phosvitin (3). β-Lactoglobulin forms complexes with casein (4) and α-lactalbumin (5).

In preliminary gel filtration experiments at low ionic strength in the pH range 6.5 to 8.5, cytochrome *c* was eluted with difficulty from Bio-Gel P-100,[3] and the addition of β-lactoglobulin in the same buffer noticeably increased the rate at which cytochrome *c* was eluted. In addition, the proportion of ferrocytochrome *c* in the eluate increased relative to that applied to the column.

These findings encouraged us to study the nature of the complex formed between the two proteins and to investigate the apparent reduction of ferricytochrome *c* in the presence of β-lactoglobulin.

MATERIALS AND METHODS

Bovine β-lactoglobulin AB (mixture, 3× crystallized) generously donated by Dr. M. P. Thompson of this laboratory was used for most of this study.

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[3] Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Samples of pure genetic variants A, B, and C and the analogous swine protein type AA were used for comparison. Horse heart cytochrome *c* (A grade, salt-free) was obtained from Calbiochem. Two different lots of cytochrome *c* containing 4 or 7% reduced iron behaved identically in these experiments and were used without further purification. All other chemicals used were the best available commercial grades.

Fresh protein solutions were prepared immediately before use to minimize the effect of slow denaturation of β-lactoglobulin (6). Cytochrome *c* concentrations were determined spectrophotometrically, using a millimolar absorptivity of 11.0 mM^{-1} cm^{-1} at the 526.5-nm isosbestic point (7). An absorptivity of 0.95 ml mg^{-1} cm^{-1} at 278 nm and a monomer molecular weight of 18,000 (8) were used to calculate molar concentrations for β-lactoglobulin.

Spectra were obtained with a Cary 14 recording spectrophotometer; cylindrical tandem cells (pathlength, 1 cm/section) were used for recording difference spectra (9) and 1-cm-pathlength cuvettes were used for all other measurements.

Sedimentation velocity was measured at 59,780 rpm and approximately 20°C using a Spinco Model E analytical ultracentrifuge equipped with Schlieren optics. Sedimentation patterns, at a constant phase plate angle of 60°, were photographed on spectroscopic plates (Type 1-N, red sensitive) using a Wratten #25 filter. For most experiments, two cells with 12-mm Kel-F single-sector centerpieces, one with a 1° positive window, were used. A mixture of the two proteins was placed in one cell, and one of the proteins at its concentration in the mixture in the other. This technique increased the comparability of the determinations by eliminating minor variations in temperature and rotor speed for two separate runs (10). Apparent sedimentation coefficients were calculated from the slope of the straight line (fitted by the method of least squares) of the logarithm of the boundary position vs time. The observed sedimentation coefficients were corrected for differences between the exact rotor temperature and 20°C, but were not corrected for the presence of buffer salts, as only relative changes were of interest (3).

The temperature dependence of the reduction of ferricytochrome *c* in the presence of β-lactoglobulin was studied in 50 mM Tris-HCl[4] at pH 8.2. At least two sets, consisting of six to eight individual experiments, were performed at each temperature using a series of cytochrome *c* concentrations (0.01 to 0.17 mM) with molar ratios of β-lactoglobulin (calculated from the monomer weight) to cytochrome *c* between 0 and 12. Solutions were incubated in a water bath at 5, 10, 15, 20, 25, or 30 ± 0.05°C. The visible spectrum between 570 and 500 nm of each sample was recorded immediately upon mixing and at timed intervals thereafter. Concentrations of ferro- and ferricytochrome *c* were calculated, using the following millimolar absorptivities: 15.9 at 520 nm and 27.7 at 550 nm for ferrocytochrome *c*; 10.2 at 520 nm (7) and 8.5 at 550 nm (11) for ferricytochrome *c*. A check of the total cytochrome *c* concentration was also obtained from these spectra.

The free sulfhydryl group of β-lactoglobulin was derivatized with *N*-dansylaziridine as described by Scouten *et al.* (12). The emission spectrum before and after incubating the derivatized β-lactoglobulin with cytochrome *c* was recorded, using an Aminco Bowman spectrophotofluorimeter.

RESULTS

Sedimentation velocity. The sedimentation velocity patterns for a series of varying molar ratios of β-lactoglobulin and cytochrome *c* in 50 mM Tris–HCl at pH 8.2 are illustrated in Fig. 1. A and G represent solutions of cytochrome *c* and β-lactoglobulin, respectively. All patterns for mixtures of the two proteins show some material moving more rapidly than either protein alone. When the ratio of cytochrome *c* to β-lactoglobulin is greater than 1, a slower boundary indicating uncomplexed cytochrome *c* is also observed (B). At molar ratios of 1 and 0.5 (C,D) a single relatively sharp, symmetrical boundary is seen; the rate at which this boundary traverses the cell decreases with increasing relative concentration of β-lactoglobulin. At a 0.3 ratio (cytochrome *c*:β-lactoglobulin) the boundary (E) has a flattened appearance and moves slower than the boundaries obtained at lower ratios. A 0.17 molar ratio shows a major boundary (F) moving at the same velocity as that of β-lactoglobulin, but with a leading shoulder. The sedimentation patterns indicate that under these conditions, complex formation has occurred.

Figure 2 shows the variation in sedimentation coefficient with molar ratio of cytochrome *c* to β-lactoglobulin. At low ratios, the apparent sedimentation coefficient approaches that of β-lactoglobulin.

[4] Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH adjusted with sodium hydroxide; Tris–HCl, 2-amino-2-hydroxymethyl-1,3-propanediol, pH adjusted with HCl.

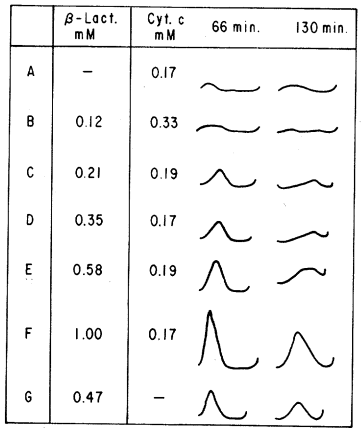

FIG. 1. Sedimentation velocity patterns of β-lactoglobulin–cytochrome *c* mixtures. All solutions are 50 mM in Tris–HCl at pH 8.2. The earliest patterns shown here were photographed 66 min after attaining full speed of 59,780 rpm and about 90 min after preparation and mixing of the protein solutions. Patterns were traced directly from the photographic plates.

As the relative concentration of cytochrome *c* is increased, the sedimentation coefficient also increases, reaching a constant value (s_{20} = 3.7) at a molar ratio slightly greater than 1. At higher ratios a second boundary due to free cytochrome *c* is measurable. In the experiment used for Fig. 1B the area of the slower peak (s_{20} = 1.5) was 68% of that for the same cytochrome *c* concentration without added β-lactoglobulin.

The apparent sedimentation coefficient (s_{20} = 2.03 ± 0.06) obtained here for β-lactoglobulin at concentrations between 0.13 and 1.0 mM agrees reasonably with values of about 2.1 each for β-lactoglobulin A and B under similar conditions as reported by Zimmerman *et al.* (13). The value of s_{20} = 1.5 ± 0.1 at pH 8.2 obtained here for cytochrome *c* at concentrations between 0.07 and 0.30 mM is in line with the published values $s_{20,w}$ = 1.83 at pH 7.0 (14) and s_{20} = 1.7 at pH 7.5 (3).

Dialysis of protein mixtures against the buffer for 24 h did not change the sedimentation patterns obtained. Increased ionic strength (0.2 M sodium chloride) caused the appearance of a slower shoulder on the main boundary for a cytochrome *c* and β-lactoglobulin mixture at a 0.63 molar ratio. While the sedimentation coefficient (s_{20} = 2.6) for the major boundary is larger than that for either isolated protein, it is significantly smaller than that (s_{20} = 3.4) obtained for the same protein mixture without the added salt. Although the slower shoulder is not well resolved, its sedimentation rate approximates that for β-lactoglobulin alone, and there is a hint of a second slow boundary corresponding to uncomplexed cytochrome *c*. These results suggest that increasing the ionic strength of the solution interferes with complex formation.

Spectral observations of the complex. When solutions of β-lactoglobulin and cytochrome *c* are examined by difference spectrophotometry under conditions that indicate complex formation in the ultracentrifuge, there is initially no change in the heme spectrum due to the mixing of the two proteins. The difference spectrum of these mixtures (Fig. 3) does change with time. The presence of isosbestic points at 556, 541.5, 527, and 504 nm (7) indicates that ferri- and ferrocytochrome *c* are the only species absorbing in the visible region. The shift of the Soret band to longer wavelength and the increasing absorbances at 520 and 550 nm indicate a conversion of ferri- to ferrocytochrome *c*, in the presence of β-lactoglobulin, which is greater than any autoreduction occurring in the unmixed solutions. When the

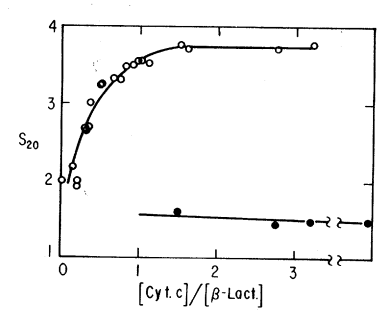

FIG. 2. Variation of the sedimentation coefficient with molar ratio of cytochrome *c* to β-lactoglobulin in the reaction mixture. Concentrations are 0.08 to 0.35 mM for cytochrome *c* and 0.1 to 0.6 mM for β-lactoglobulin. The upper curve (○) represents s_{20} for β-lactoglobulin and complex. The lower curve (●) represents unbound cytochrome *c* at these ratios, or cytochrome *c* alone at the far right.

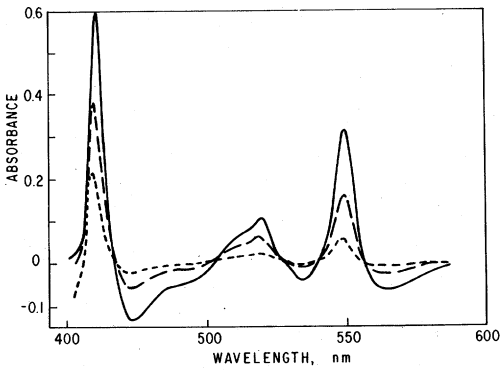

FIG. 3. Variation with time of the difference spectrum of a mixture of β-lactoglobulin and cytochrome *c* against unmixed solutions. Equal volumes of 0.34 mM β-lactoglobulin and 0.11 mM ferricytochrome *c* solutions in 50 mM Tris–HCl, pH 8.2 were mixed. Incubation was at room temperature, although for this experiment the β-lactoglobulin solution was refrigerated overnight before mixing with the cytochrome *c*. The time elapsed between mixing and the start of the scan was 2 min (- - -), 32 min (– –), and 285 min (——). The wavelength scale is compressed in the Soret region.

cytochrome *c* concentration is sufficiently high, the weak 695-nm band characteristic of a native conformation of ferricytochrome *c* can be seen in mixtures with β-lactoglobulin. This band decreases with time, but does not disappear until the reduction of ferricytochrome *c* is nearly complete. Both the rate and the final extent of reduction of ferricytochrome *c* depend on pH, specific buffer salts, ionic strength, and temperature as well as protein concentration.

Buffer effects. Below pH 8, the rate of reduction of ferricytochrome *c* by β-lactoglobulin is too slow to be measured conveniently. At pH 8 and 15°C, using matching protein solutions, 10% conversion of ferri- to ferrocytochrome *c* is observed after 26 min in sodium phosphate, 4.7 min in Hepes, and 2.4 min in Tris–HCl. After 24 h, the amount of ferrocytochrome *c* is 17% in phosphate, 20% in Hepes, and 48% in Tris–HCl, and similar dilutions of cytochrome *c* without β-lactoglobulin contain 10% ferrocytochrome *c* irrespective of the buffer salt. Thus, the specific buffer salt affects both the rate and the final extent of ferricytochrome *c* reduction in the same direction. Aggregates detectable in the ultracentrifuge were formed in each of these buffers.

The effects of buffer concentration and ionic strength on the reduction of ferricytochrome *c* were examined in solutions of Tris–HCl at pH 8.2 and 15°C. The optimum buffer concentration was found to be 50 mM; at lower concentrations the pH decreased as the iron was reduced. Much higher buffer concentrations or the addition of neutral salts caused a marked decrease in reaction rate, as well as the interference with complex formation noted above.

The reduction of ferricytochrome *c* in the presence of β-lactoglobulin was studied in detail at pH 8.2, 50 mM Tris–HCl at 15°C. Figure 4 shows the change in ferrocytochrome *c* concentration with time when the β-lactoglobulin concentration is varied. A twofold variation in total cytochrome *c* concentration does not affect the initial linear portion of these curves when the β-lactoglobulin concentration is held constant. The reaction is essentially complete in 24 h. When the concentration of β-lactoglobulin is twice as great as that of cytochrome *c*, there is little increase in ferrocytochrome *c* between 24 and 48 h other than that which occurs during this time period in the absence of β-lactoglobulin. At higher relative β-lactoglobulin concentrations the ferrocytochrome *c* con-

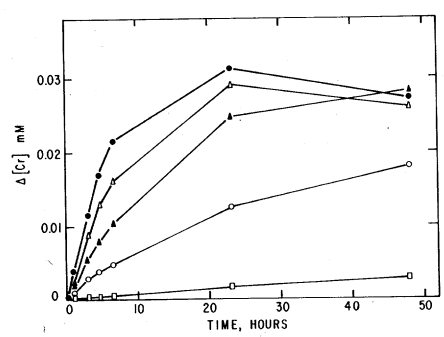

FIG. 4. Change in the concentration of ferrocytochrome *c* with time as a function of the amount of β-lactoglobulin added. The total cytochrome *c* concentration was 0.047 mM; the initial ferrocytochrome *c* concentration was 0.0025 mM. β-Lactoglobulin concentrations were: □, 0; ○, 0.05; ▲, 0.11; △, 0.22; and ●, 0.54 mM. Incubation was at 15°C.

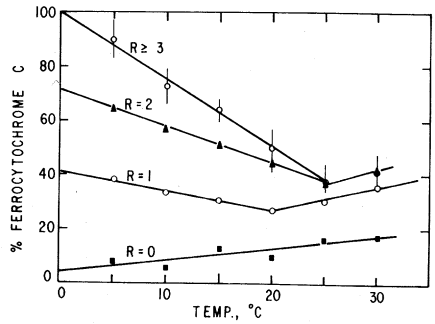

FIG. 5. Effects of temperature and molar ratio of β-lactoglobulin to cytochrome *c* on the percentage in the ferrocytochrome *c* form after 24 h. R = [β-lactoglobulin]/[cytochrome *c*].

centration decreases slightly after the first 24 h. Curves obtained at 5, 10, 20, 25, and 30°C are qualitatively similar although differing in the amount of reduction occurring.

The extent of ferricytochrome *c* reduction, 24 h after the initial mixing was studied as a function of temperature and concentration of β-lactoglobulin (Fig. 5). When the concentration of β-lactoglobulin was at least three times as great as that of cytochrome *c*, the extent of iron reduction after 24 h decreased linearly with increasing temperature between 5 and 25°C. A small increase in ferrocytochrome *c* concentration is noted at 30°C. Similar trends with flatter curves and a shift in the temperature minimum are observed as the concentration of β-lactoglobulin is lowered toward that of cytochrome *c*. Some iron reduction occurs in the absence of β-lactoglobulin, in which case the amount of ferrocytochrome *c* increases with increasing temperature but does not exceed 17%.

While the extent of reduction after 24 h depends on the relative concentrations of the two proteins, the initial rates of iron reduction are more sensitive to actual concentration of β-lactoglobulin. As seen in Fig. 4, the concentration of ferrocytochrome *c* increases in a linear fashion for several hours. Plots based on the assumption of first- or higher-order reaction with respect to cytochrome reduction were less linear. Comparison of initial rates of reduction at varying β-lactoglobulin concentrations gives a reaction order of 1.1 ± 0.5 with respect to the β-lactoglobulin.

Dialysis of β-lactoglobulin against cytochrome *c* was totally ineffective in reducing the iron. The spectrum of a solution of cytochrome *c* which had been dialyzed against the 50 mM Tris–HCl buffer at 5°C for 16 h was identical to that of a second aliquot which had been dialyzed against a fivefold molar excess of β-lactoglobulin.

Figure 6 shows the temperature dependence of the initial rate constant. The initial rate of increase in concentration of ferrocytochrome *c* in these experiments was dependent on the concentration of β-lactoglobulin but not on the concentration of ferricytochrome *c*. In contrast to the 24-h reduction, the initial rate shows a minimum at 15°C.

The individual genetic variants A, B, and C of bovine β-lactoglobulin also formed complexes with cytochrome *c* and were able to reduce the iron; temperature dependence for iron reduction was qualitatively the same as with β-lactoglobulin AB, but the actual rate depended on the particular variant used. Both the initial rate of iron reduction and the total reduction in 24 h at moderate temperatures could be significantly increased by using β-lactoglobulin which had been pretreated in pH 8.2 buffer several hours before being added to the cytochrome *c*. While room temperature storage of the β-lactoglobulin solution was effective, low temperature storage, which favors a conformational

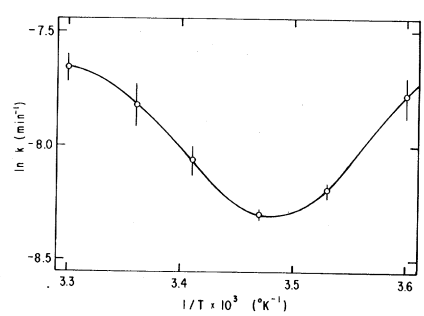

FIG. 6. Initial rate of iron reduction as a function of temperature. Concentration ranges were cytochrome *c* (0.01–0.17 mM) and β-lactoglobulin (0.01–2.0 mM). The initial rate constant, $k = (d[Fe^{2+}]/dt)[\beta\text{-lactoglobulin}]^{-1}$.

change, increased the initial rate even more. The difference spectrum (Fig. 3) was obtained with a β-lactoglobulin solution that had been aged for 16 h at 4°C prior to being mixed. This treatment doubled the percentage of ferrocytochrome *c* obtained in 24 h and increased the initial rate by a factor of 4 when the reaction with cytochrome *c* was carried out at 25°C. Any conformational changes or specific groups involved in this reaction must be common to all the variants of β-lactoglobulin. Neither the initial rate nor the final concentration of ferrocytochrome *c* obtained was changed by the use of previously stored cytochrome *c* solutions, nor when completely oxidized cytochrome *c* was used.

The emission spectrum of the *N*-dansylaziridine derivative of β-lactoglobulin consisted of a single peak of 485 nm when excited at 345 nm. When cytochrome *c* was mixed with this derivative, there was an immediate decrease in the fluorescent intensity. The rate of reduction of ferricytochrome *c* by this derivative was about 10% of that by a comparable nonderivatized β-lactoglobulin solution. After 24 h, the β-lactoglobulin–dansylaziridine derivative produced the same emission spectrum as when freshly prepared, but that mixed with cytochrome *c* had a greatly reduced intensity due to quenching by the cytochrome *c* and showed a shoulder at 535 nm attributable to the fluorescence of unbound dansylaziridine (Fig. 7).

Reduction of ferricytochrome *c* was also accomplished using cysteine or cysteine·HCl. In these cases the reduction was more complete and much more rapid than with β-lactoglobulin but occurred only at or above pH 8.9, which is near the p*K* for nonprotein sulfhydryl groups (p*K* = 9.1).

Swine β-lactoglobulin, which has no free sulfhydryl groups, formed a complex with cytochrome *c*, as demonstrated by sedimentation velocity. However, the swine protein was a very poor reducing agent, resulting in 13% reduced cytochrome iron under conditions where bovine β-lactoglobulin gave 47% reduction and autoreduction alone gave 10%.

DISCUSSION

The results presented here indicate the formation of a complex of limited size between cytochrome *c* and β-lactoglobulin in solutions of low ionic strength and moderate pH. Although polymers of cytochrome *c* are known (14), they are of indefinite size and would not be expected to occur under the conditions of these experiments. Additionally, the sedimentation patterns of solutions containing cytochrome *c*, either alone or at a higher molar concentration than β-lactoglobulin, do not indicate the presence of a cytochrome *c* species other than the monomer. McKenzie (15) reviewed the molecular sizes and conformations of β-lactoglobulin as a function of pH. The dissociation studies of Georges *et al.* (16) suggest that our solutions of β-lactoglobulin are 65 to 70% monomer. Also, β-lactoglobulin undergoes a conformational change beginning at pH 7.5 that appears to proceed via the monomer (17). The effects of other proteins on the dissociation and conformational equilibria of β-lactoglobulin have not yet been examined.

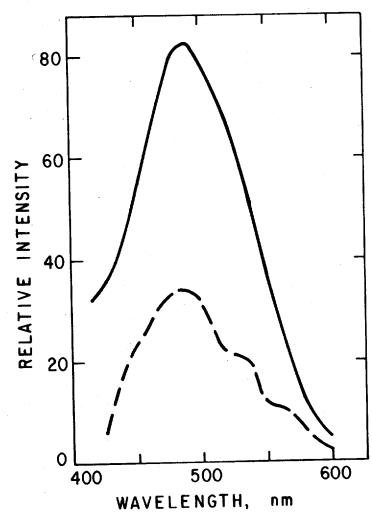

FIG. 7. Fluorescent emission spectra of the *N*-dansylaziridine derivative of β-lactoglobulin (——), and the reaction mixture, containing the same concentration of derivative, 16 h after adding cytochrome *c* (– –). The β-lactoglobulin to cytochrome *c* ratio was 1.6, and the reaction was carried out at 15°C; excitation was at 345 nm.

Complex formation between proteins having very different isoionic points such as cytochrome *c* and β-lactoglobulin (10.6 and 5.3, respectively) is probably electrostatically controlled, although these proteins have hydrophobic areas which may interact. These modes of interaction are consistent with the complex formation observed here.

Although the maximum sedimentation coefficient is found when the starting mixture contains a slight excess of cytochrome *c*, the presence of free cytochrome *c* in these mixtures suggests that the complex consists of one cytochrome *c* molecule per β-lactoglobulin monomer. Electrostatic considerations suggest that cytochrome *c* could bind to either the monomer or the dimer. Thus the complex may exist as an equilibrium mixture of 30,000 to 60,000-molecular weight species, depending on the dissociation constant for β-lactoglobulin under these conditions. Strictly electrostatic interactions would not generally lead to the formation of discrete, small complexes. However, several studies of the interactions of cytochrome *c* with other proteins have shown that 1:1 complexes form with globular proteins such as cytochrome oxidase (1) or cytochrome peroxidase (2); a 1:1 reaction, reported by Wu *et al.* (18), occurs between cytochrome *c* and myoglobin, but this complex could not be isolated. Yoshimura *et al.* (19) have recently reported a 2:1 complex of cytochrome *c* with yeast L-lactate dehydrogenase, a four-subunit enzyme which might have been expected to bind four molecules of cytochrome *c*. A complex of cytochrome *c* and phosvitin at ratios between 10:1 and 30:1 has been reported by Taborsky (3); phosvitin, being an elongated fibrous protein, may provide more binding surface, as well as a large number of highly negatively charged sites due to its phosphate content. A 3:1 complex of β-lactoglobulin with κ-casein (not electrostatic) has been reported by Tessier *et al.* (20), but again, casein is not a globular protein.

In this study, the heme portion of cytochrome *c* does not seem to be directly involved in the complex formation with β-lactoglobulin. Initial complex formation is very fast since complexing immediately after mixing the two proteins can be demonstrated by gel chromatography and by ultracentrifugation. However, spectra obtained immediately upon mixing showed no changes in the heme due to the presence of β-lactoglobulin. Since reduction of the iron is prevented when the proteins are separated by dialysis membrane and inhibited by salt concentrations high enough to interfere with complex formation, the following scheme is proposed:

$$C_0 + \beta\text{-lact.} \overset{\text{fast}}{\rightleftharpoons} C_0 - \beta\text{-lact.} \overset{\text{slow}}{\rightleftharpoons} C_0 - \beta\text{-lact.}^* \overset{\text{fast}}{\rightleftharpoons} C_r\ \beta\text{-lact.}^*$$

where C_0, C_r, β-lact., and β-lact.* represent ferricytochrome *c*, ferrocytochrome *c*, β-lactoglobulin, and conformationally changed β-lactoglobulin, respectively.

The reduction of ferri- to ferrocytochrome *c* occurs only after the complex has formed and is more sensitive to particular buffer salts than is complex formation, suggesting that different amino acid groups are involved in the two interactions. Greenwood and Palmer (21) reported similar inhibition due to phosphate when reducing ferricytochrome *c* with ascorbate. It appears to be a kinetic effect as the redox potentials determined in Tris and phosphate buffers by Margalit and Schejter (22) were identical. In a second paper, Margalit and Schejter (23) reported the binding of two chloride or two phosphate anions per molecule of ferricytochrome *c*; one sulfanilate anion could be bound, but Tris–cacodylate had an anti-binding effect. If the inhibition to iron reduction is due to steric problems in positioning the donor and acceptor, then the two phosphate ions would be expected to be more effective than the two chlorides that might be available from the Tris–HCl. Hepes would be expected to behave somewhat like the sulfanilate anion with respect to ferricytochrome *c*, and it may also interact with the β-lactoglobulin, which binds other large sulfonates, namely, methyl orange (24), *n*-octylbenzene-*p*-sulfonate (25), and dodecyl sulfate, the binding of which was found to interfere with the oxidation of β-lactoglobulin by ferricy-

anide (26). Tris–HCl must either be neutral toward the two proteins or interact cooperatively with them, since both the rate and the extent of iron reduction are enhanced.

The reduction of ferricytochrome *c* by dithionite (27), ferrohexacyanide (28), or ascorbate (21) in the pH 7–11 range proceeds as a two-step reaction, with both steps being complete in a matter of seconds. Rapid reduction of ferricytochrome also occurs in the presence of cytochrome reductase, xanthine oxidase, and myoglobin, each with a pH optimum between 7 and 7.5. By comparison, the reduction of ferricytochrome *c* in the presence of β-lactoglobulin occurs only above pH 7.5 and is a much slower reaction. The type of two-step reduction due to isomerization of cytochrome *c* in alkaline solutions is not seen here. The changes in the rate of reduction with time (Fig. 4) and the difference in optimum temperature for initial rate and total percentage reduction (Figs. 5 and 6) suggest that the mechanism, or at least the rate-determining step, changes with time. Hence, the rate of iron reduction is determined by those changes in size and conformation of β-lactoglobulin by which electrons can be transferred from this protein.

The temperature dependence of the rate of iron reduction parallels the rate of alkaline denaturation of β-lactoglobulin (29), indicating a similar rate-determining step for the two reactions. The analogy may not be exact, since the denaturation studies were conducted at pH 9.3 and were irreversible while the conformational change at pH 8.2 is reversible (17). In either case, the change involves the dissociation of β-lactoglobulin into monomer units with the opening up of a hydrophobic region and an increased reactivity of the free sulfhydryl group. It may be that the presence of cytochrome *c* with its hydrophobic area favors the dissociation of the β-lactoglobulin to a greater extent than normal at this pH, if the complex formed allows the two hydrophobic areas to interact. Both the sulfhydryl group of β-lactoglobulin and the heme group of cytochrome *c* are in the hydrophobic portions of their respective proteins. Thus, if the hydrophobic regions are interacting with each other, these two groups should be in close proximity. The pH dependence of iron reduction is similar to that for the displacement of the sulfhydryl hydrogen of β-lactoglobulin by mercury (30). The inability of swine β-lactoglobulin, which contains no free sulfhydryl groups, to reduce cytochrome iron and the diminished reactivity of the *N*-dansylaziridine–β-lactoglobulin derivative with cytochrome *c* also suggest that the sulfhydryl group is the electron source. It is of particular interest that the *N*-dansylaziridine can be displaced somewhat from the sulfhydryl group if ferricytochrome is mixed with the complex, allowing some iron reduction to occur.

The similarity in redox potentials of cytochromes (−300 to +400 mV) (31) and glutathione (−350 to +40 mV) (32) suggests that reduction may occur through the direct interaction of a sulfhydryl group on β-lactoglobulin with the heme of cytochrome rather than through a chain of aromatic residues in the proteins (31).

The enhanced reduction of cytochrome *c* by alkaline-pretreated β-lactoglobulin suggests that the sulfhydryl group may be frozen in an open position. However, the similarity in the sedimentation patterns of these complexes with those using untreated β-lactoglobulin suggests that there is not a large difference in overall shape of the molecule. The tendency toward reoxidation that is seen with complexes prepared from either fresh or alkaline pretreated β-lactoglobulin solutions may mean that cytochrome *c* stabilizes a configuration in which the β-lactoglobulin can form disulfide bonds. The amino acid substitutions in the different genetic forms of β-lactoglobulin apparently do not affect its complex forming ability and have only a minor effect on iron reduction.

Although no physiological significance can be attached to complex formation between such unrelated proteins as β-lactoglobulin and cytochrome *c*, this interaction may serve as a model in studying the behavior of either protein in the appropriate physiological system. Complex forma-

tion did not depend on iron reduction, occurring between β-lactoglobulin and either ferri- or ferrocytochrome *c*. Iron reduction was dependent on complex formation and on the availability of a sulfhydryl group from the β-lactoglobulin.

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