THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 257, No. 9, Issue of May 10, pp. 4783–4787, 1982
*Printed in U.S.A.*

# Association of Cytochrome $b_5$ and Cytochrome P-450 Reductase with Cytochrome P-450 in the Membrane of Reconstituted Vesicles*

(Received for publication, December 3, 1981)

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**A protein-protein association of cytochrome P-450 $LM_2$ with NADPH-cytochrome P-450 reductase, with cytochrome $b_5$, and with both proteins was demonstrated in reconstituted phospholipid vesicles by magnetic circular dichroism difference spectra. A 23% decrease in the absolute intensity of the Soret band of the magnetic CD spectrum of cytochrome P-450 was observed when it was reconstituted with reductase. A difference spectrum corresponding to a 7% decrease in absolute intensity was obtained when cytochrome $b_5$ was incorporated into vesicles that already contained cytochrome P-450 and cytochrome P-450 reductase compared to a decrease of 13% in absolute intensity when cytochrome $b_5$ was incorporated into vesicles that contained only cytochrome P-450. The use of magnetic circular dichroism confirmed that protein-protein associations that have been detected by absorption spectroscopy between purified and detergent-solubilized proteins also exist in membranes. High ionic strength was shown to interrupt direct electron flow from cytochrome P-450 reductase to cytochrome P-450 but not the electron flow from reductase through cytochrome $b_5$ to cytochrome P-450. Upon incorporation of cytochrome $b_5$ into cytochrome P-450- and cytochrome P-450 reductase-containing vesicles, an increase of benzphetamine *N*-demethylation activity was observed. The magnitude of this increase was numerically identical to the residual activity of the reconstituted vesicles measured in the presence of 0.3 M KCl. It is concluded that there is a requirement for at least one charge pairing for electron transfer from reductase to cytochrome P-450. These observations are combined in a proposed mechanism of coupled reversible association reactions in the membrane.**

The endoplasmic reticulum of liver cells is known to contain at least two major electron transport systems. One of them transfers electrons from NADH for fatty acyl desaturation (1, 2) and the other utilizes reducing equivalents from NADPH for a variety of reduction and hydroxylation reactions involving cytochrome P-450 systems (3, 4). In both systems cytochrome $b_5$ seems to be involved as an electron carrier. The cytochrome P-450 and cytochrome P-450 reductase system clearly is functional without cytochrome $b_5$ for the majority of catalyzed reactions. However, the addition of cytochrome $b_5$ has been shown to result in higher metabolic activity (5–7) in a variety of systems. Therefore, speculations about its facilitory or even regulatory role in a liver cell might seem reasonable. If cytochrome $b_5$ acts as a link between these two electron transport systems, it is possible that there is such a regulation, depending on availability of NADPH or NADH. An understanding of the mechanisms of interaction of these three proteins would lead to improved model systems for the endoplasmic reticulum which could be used for studies of drug metabolism.

With the availability of purified proteins (8, 9), it has become possible to reconstitute them into functional units to study drug metabolism (10–13) as well as aspects of the mechanism of protein-protein interactions (14). Evidence was obtained for a binary complex formation (14, 15) between cytochrome P-450 $LM_2$ and NADPH-cytochrome P-450 reductase (referred to as cytochrome P-450 and reductase, respectively). These studies were performed with micelle-reconstituted systems that contain only about 20 phospholipid molecules/protein. Therefore, it cannot be excluded that the observed interaction reflects unspecific contacts between these hydrophobic membrane proteins.

In the present paper the interaction of cytochrome P-450 and NADPH-cytochrome P-450 reductase was studied in vesicle-reconstituted systems. These contain sufficient phospholipid that no protein-protein interaction would be induced solely to prevent exposure of hydrophobic protein surfaces to the aqueous environment. Furthermore, cytochrome $b_5$ was included in order to study a more complete NADPH-dependent electron chain. The bilayer of these vesicles is composed of a 2:1 mixture of phosphatidylcholine and phosphatidylethanolamine which was shown to provide functional and structural similarity to microsomes (13, 16). Complex formation in the membrane was studied by magnetic CD. Magnetic CD has been shown to provide useful information about the electronic structure at the fifth ligand position, the substrate binding site, the oxidation state, and the spin state of cytochrome P-450 in microsomes (17) and purified cytochrome P-450 in reconstituted systems (18, 19). Enzymatic measurements were used to study the stimulatory role of cytochrome $b_5$ and to obtain information on the electrostatic nature of these protein-protein interactions. The data support a model of specific reversible association reactions in a two-dimensional phospholipid bilayer in which electrostatic interactions are involved.

## MATERIALS AND METHODS

*Materials*—Cytochrome P-450 $LM_2$ was prepared from liver microsomes of phenobarbital-treated rabbits by a modification of described procedures (8, 9). Cytochrome P-420, which was present at about 5% after purification, could no longer be detected after reconstitution in vesicles. NADPH-cytochrome P-450 reductase was prepared from the same microsomes by affinity chromatography (20). Its

* This research was supported by a grant from the National Institute of Occupational Safety and Health (OH 00978). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a stipend from the Alexander von Humboldt-Stiftung.

activity toward cytochrome *c* was 37–40 μmol/min × mg of protein at 30 °C in 0.3 M potassium phosphate buffer containing 20% glycerol and 0.5% cholate. No cytochrome P-450 reductase was detectable in the cytochrome P-450 preparations and *vice versa*. Egg phosphatidylcholine and phosphatidylethanolamine were prepared from 2-day-old eggs by the method of Singleton *et al.* (21) with special precaution to do all manipulations under an atmosphere of nitrogen. Sodium cholate was purchased from Calbiochem-Behring and recrystallized from ethanol/water before use.

*Reconstitution into Phospholipid Vesicle*—Cytochrome P-450 $LM_2$ and cytochrome P-450 reductase were reconstituted by the previously described cholate dialysis technique (3, 13, 19) with slight modifications. No difference in technique or protein:lipid ratios was made whether the two proteins were individually or co-reconstituted. An aliquot of a lipid mixture of 66 mg of egg phosphatidylcholine and 33 mg of phosphatidylethanolamine dispersed in 1 ml of 20% sodium cholate in water was added to the protein solution to give the desired lipid:protein ratio. Solutions of cytochrome P-450 and cytochrome P-450 reductase were combined in the case of the co-reconstitution before the dissolved lipid was added. The protein concentration was adjusted to 0.5 mg/ml by addition of the same 0.3 M potassium phosphate buffer. The final sodium cholate concentration was 2%. This starting mixture was allowed to equilibrate overnight under a $N_2$ atmosphere at 4 °C. The sodium cholate was then removed at 4 °C during 3 days by dialysis against 0.02 M potassium phosphate buffer, pH 7.5, containing 20% glycerol, 0.1 mM dithiothreitol, and 0.1 mM EDTA with at least 7 changes of buffer.

*Analysis of Reconstituted Vesicles by Density Gradient Centrifugation*—Linear density gradients were prepared from 5–50% glycerol in the dialysis buffer, such that the placement of 1.5 ml of a vesicle suspension in 20% glycerol in the gradient filled the tube. They were spun for 16–22 h at $10^5 \times g$ at 4 °C. The density of vesicles that formed a band in a gradient was calculated from their distance to the bottom of the tube. The co-reconstituted vesicles with cytochrome P-450 and cytochrome P-450 reductase with a lipid:protein ratio of 5:1 (w/w) formed a band at density 1.06. Similar gradients demonstrated incorporation of solubilized, detergent-free cytochrome $b_5$ into vesicle.

*Measurements*—Vesicle preparations were used for measurements within a day after dialysis. All measurements of magnetic CD and enzymatic activity were carried out in 0.02 M potassium phosphate buffer, pH 7.5, containing 20% glycerol. Formaldehyde production was determined by the Nash reaction (22). The final concentrations were 0.2 μM cytochrome P-450, 2 mM NADPH, and 1 mM benzphetamine. The reactions were linear for 10 min at 30 °C. For the experiments at higher ionic strength, the same buffer was used and 0.3 M KCl was present during thermostatting for 10 min before addition of NADPH.

A Cary 219 spectrometer was used for absorption measurements. Matched tandem cuvettes with inner compartments of 4-mm diameter each were used for difference spectroscopy measurements. These measurements were carried out in 0.1 M potassium phosphate buffer, pH 7.5, containing 20% glycerol and 0.2% sodium cholate.

All magnetic CD spectra were recorded on a Japan Spectroscopic Company spectropolarimeter (JASCO model J-40) containing a 15.0-kG electromagnet with the field direction parallel to the direction of light propagation. The magnetic CD spectral data shown have been corrected for natural CD (magnetic $CD_{obs}$ = CD + magnetic CD). All magnetic CD spectra were recorded at 20 °C. The data are reported in terms of molar magnetic ellipticity, $[\theta]_M$, in the units degree $cm^2$ $dmol^{-1}$ $G^{-1}$. The data were recorded, normalized, smoothed, and manipulated on a Nova 840 computer and stored on magnetic tape. The solutions were made identical in the concentrations of cytochrome P-450 and cytochrome P-450 reductase and measured in the same cuvette in the same orientation. The magnetic CD difference spectra with cytochrome $b_5$ were obtained with the use of tandem cuvettes. One of the (4-mm inner diameter) compartments contained the vesicle suspension and the other a solution of detergent-free cytochrome $b_5$. After a spectrum had been measured, the contents of the two compartments were mixed by inverting them into a central compartment of the cuvette, allowed to equilibrate for 30 min at 21 °C, and measured again. Difference spectra were obtained by computer subtraction of magnetic CD spectra. The magnetic CD of reconstituted cytochrome P-450 reductase was no more than 9% of the maximal intensity of cytochrome P-450 at any wavelength and was negligible above 520 nm.

## RESULTS

Absorption difference spectroscopy was used to demonstrate an interaction of cytochrome P-450 with NADPH-cytochrome P-450 reductase as well as with cytochrome $b_5$. The detergent-solubilized proteins of the purity described under "Materials" were used. From the association reaction with reductase a difference spectrum was obtained that was nearly identical to the one reported previously (14). The possibility of an interaction between cytochrome P-450 and cytochrome $b_5$ was tested by the same technique. Upon mixing of the two solutions in the sample cell a difference spectrum developed with a half-time of about 2 min (Fig. 1). This association reaction is faster than the one with reductase, but the difference in rate might be artifactual, because the aggregation states of these detergent-solubilized proteins could be different.

The possibility that the difference spectrum in Fig. 1 is a fortuitous result of different concentrations or volumes in the cuvette can be ruled out because no difference spectrum was seen when subsequently the reference cuvette was mixed. Furthermore, the time-dependent formation is indicative of a reaction. In addition, the difference spectra that resulted from different concentrations of cytochrome P-450 and cytochrome $b_5$ were very different. A dissociation of the complex between cytochrome P-450 and cytochrome $b_5$ was produced by the addition of KCl to a 0.3 M final concentration. A slow reaction was observed with a half-time of about 24 min.

The absorption difference spectroscopy described above was also carried out in order to relate the following magnetic CD measurements of vesicle-reconstituted systems to other established data on cytochrome P-450 interactions with reductase. Magnetic CD spectroscopy was chosen over UV/visible absorption spectroscopy because of its insensitivity to changes in light scattering in vesicle preparations. A change in a magnetic CD spectrum reflects a change in the interactions of the porphyrin. The magnetic CD spectrum of reductase was found to be much less intense than that of cytochrome P-450 and to be very broad. Therefore, it can be assumed that it contributes very little to the observed magnetic CD difference spectra shown in Fig. 2.

Three different protein-protein interactions were examined in similar reconstituted vesicles. The magnetic CD difference spectrum in Fig. 2*A* was obtained by subtracting the sum of the magnetic CD spectra of individually reconstituted reductase and cytochrome P-450 from that of the co-reconstituted preparation. A decrease of 23% of the absolute intensity of the magnetic CD Soret band of cytochrome P-450 was observed. This decrease is similar to the one obtained in micelle-reconstituted systems (14) and demonstrates the existence of a

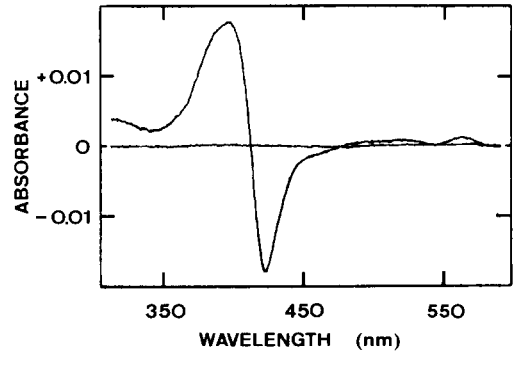


FIG. 1. **Absorption difference spectrum obtained by mixing equimolar solutions of purified and detergent-solubilized cytochrome $b_5$ and cytochrome P-450 $LM_2$.** It developed with a half-time of 2 min at 5.6 μM protein concentrations in 0.1 M potassium phosphate buffer, pH 7.5, containing 20% glycerol and 0.2% sodium cholate at 22 °C.

specific protein association in a membrane. The second magnetic CD difference spectrum (Fig. 2*B*) shows an interaction in a bilayer between cytochrome $b_5$ and cytochrome P-450. It was obtained from the magnetic CD spectra of solutions of equimolar concentrations of cytochrome P-450 vesicles and solubilized cytochrome $b_5$ before and after mixing and equilibration. It corresponds to a decrease of 13% of the absolute intensity of the Soret band. The spectra in *A* and *B* are of very similar shape. The magnetic CD difference spectrum of vesicle-co-reconstituted reductase and cytochrome P-450 before and after equilibration with cytochrome $b_5$ had the same shape but corresponds to a decrease of only 7% of the absolute intensity of the Soret band (not shown). For an interpretation of the difference spectra it is necessary to exclude a change in the magnetic CD spectrum of cytochrome $b_5$ caused by its incorporation in a membrane. For this purpose the difference spectrum in *C* has been included which shows an insignificant difference between the magnetic CD spectrum of solubilized cytochrome $b_5$ and the spectrum of cytochrome $b_5$ interacting with protein-free vesicles. No significant difference spectrum was obtained before and after mixing vesicle-reconstituted reductase with cytochrome $b_5$ (*D*). An almost identical difference spectrum as in Fig. 2*A* was obtained when the experiment was repeated with identical proteins taken from another purification batch.

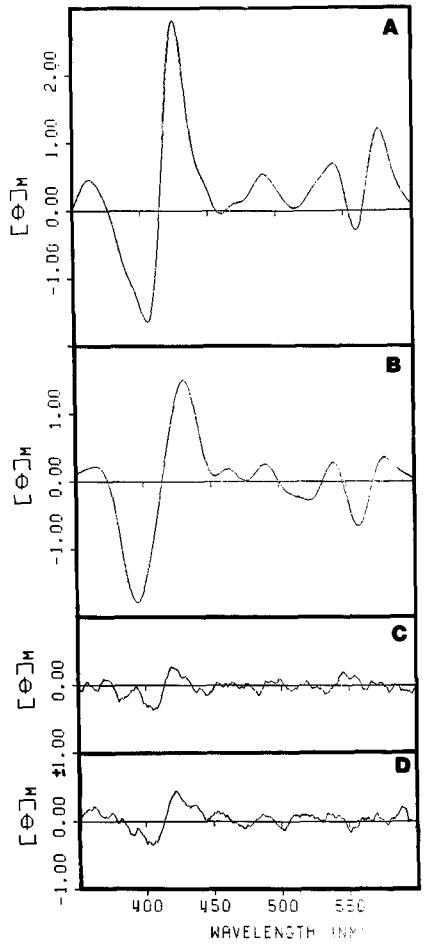


FIG. 2. **Magnetic CD difference spectra of vesicle-reconstituted systems, obtained by subtraction of magnetic CD spectra which were measured in 0.02 M potassium phosphate buffer, pH 7.5, containing 20% glycerol at 20 °C.** The spectral differences are a result of an association in the membrane of co-reconstituted cytochrome P-450 and cytochrome P-450 reductase (*A*) and cytochrome P-450 with added cytochrome $b_5$ (*B*). Incorporation of solubilized cytochrome $b_5$ in pure phospholipid vesicles gave an insignificant magnetic CD change (*C*). This was also the case following incorporation of solubilized cytochrome $b_5$ in cytochrome P-450 reductase vesicles (*D*).

TABLE I

*Effect of cytochrome $b_5$ on N-demethylation activity of cytochrome P-450 in the outer monolayer of a vesicle*

*N*-Demethylation activity from benzphetamine was measured at 30 °C in 0.02 M potassium phosphate buffer, pH 7.5, with 20% glycerol with and without 0.3 M potassium chloride by vesicle-reconstituted systems with and without cytochrome $b_5$. The largest S.D. is less than 4% of the number. It is seen that the activity increase in buffer by the introduction of cytochrome $b_5$ (44.3 − 25.2 = 19.1) is reflected in the remaining activity in buffer with KCl.

| Vesicle-reconstituted system | $[CH_2O]/[cytochrome\ P\text{-}450] \times min$ | |
|---|---|---|
| | Buffer | Buffer + KCl |
| Cytochrome P-450, cytochrome $b_5$, and reductase | 44.3 | 18.7 |
| Cytochrome P-450 and reductase | 25.2 | 0 |

The spectroscopic data described above give strong evidence for a complex formation in the membrane of cytochrome P-450 with reductase, with cytochrome $b_5$, and with both proteins at the same time. *N*-Demethylation activity with benzphetamine as substrate was used to further characterize the interaction between these membrane proteins. When a reductase vesicle suspension was mixed with a suspension of vesicle-co-reconstituted cytochrome $b_5$ and cytochrome P-450, the activity was 10.6 nmol/min/nmol of cytochrome P-450, compared to 6.6 without cytochrome $b_5$. Incorporation of cytochrome $b_5$ increased the activity of reductase- and cytochrome P-450-containing vesicles from 47 to 73 nmol of $CH_2O$/min/nmol of cytochrome P-450. When 0.3 M KCl-containing buffer was used, the benzphetamine demethylation activity decreased to 39%, which corresponds well to the percentage of cytochrome P-450 and reductase located on the outer surface of the vesicles. The activity of the remaining enzymes located on the inner monolayer approached zero as KCl diffused inside during a time course of hours. In order to test whether a similar dependence on higher ionic strength exists for the electron flow from reductase through cytochrome $b_5$ to cytochrome P-450, the same enzymatic activity was measured at 0.3 M KCl with the use of a vesicle-reconstituted system containing the three proteins at equimolar ratios. The results are summarized in Table I.

The activity of the enzyme system on the inside of the vesicle has been subtracted to clarify the effect of cytochrome $b_5$. The difference in specific activity between the two vesicle preparations with and without cytochrome $b_5$ in the absence of KCl is 19.1 nmol of $CH_2O$/min. This difference is due to the activating effect of cytochrome $b_5$. The residual activity of the complete system of the three proteins following the addition of 0.3 M KCl of 18.7 nmol/min is not significantly different from 19.1. This suggests that the increase in activity produced by cytochrome $b_5$ at low ionic strength is quantitatively present as residual activity in the presence of 0.3 M KCl.

## DISCUSSION

An interaction in a phospholipid bilayer of purified cytochrome P-450 $LM_2$ with NADPH-cytochrome P-450 reductase as well as with cytochrome $b_5$ was demonstrated by spectroscopic changes of absorption and magnetic CD of the porphyrin prosthetic groups. Similar changes have been observed in magnetic CD difference spectra resulting from an interaction of cytochrome P-450 and reductase in micelle-reconstituted systems (14). Studies of enzymatic activities and size

determinations of the micelle supported the hypothesis that the observed interaction reflects a specific 1:1 association of these two membrane proteins. An apparent dissociation constant on the order of 0.1 μM was determined (14, 15). On the other hand, such a value could be artifactual because it is derived from micelle-reconstituted systems in which pronounced protein-protein interactions could dominate. There are only about 20 phospholipid molecules/cytochrome P-450 in such a micelle, which is not even sufficient to surround a protein of molecular weight 50,000 with a single layer of lipids (23). Furthermore, cytochromes P-450 themselves are available only as oligomers after purification (14). The dissociation constant of these cytochrome P-450 molecules also has to be on the order of 0.1 μM, since they remain aggregated in dilute solutions and even under the nonequilibrium conditions of gel filtration. For these reasons it is important to demonstrate that the observed protein-protein association reactions are meaningful in that they also occur in the membrane of the endoplasmic reticulum. The present study supports such a projection in that an association in a membrane would not be expected to occur in the absence of specific contact sites. In the membrane of a vesicle, every protein molecule could be surrounded by several phospholipid layers, and no protein would be forced to interact with another protein in order to circumvent exposure of hydrophobic surface areas to water. The observed magnetic CD changes are of similar amplitude in micelles and in vesicles. The difference spectrum produced from an association of cytochrome P-450 with cytochrome $b_5$ was of similar shape as the one that resulted from an association with reductase. Therefore, the difference in molar ellipticity might originate mainly from a change of the environment of the cytochrome P-450 porphyrin.

The lack of a measurable magnetic CD change from an interaction of cytochrome $b_5$ with reductase does not exclude an interaction between these two proteins. The reduction of cytochrome $b_5$ by NADPH-cytochrome P-450 reductase (24) certainly demonstrates some interaction. Moreover, it has been shown by chemical modification of cytochrome $b_5$ that complementary charge pairing is necessary to achieve catalytically productive interactions (25).

The third protein-protein interaction that may occur is the one between cytochrome P-450 and cytochrome $b_5$. A difference spectrum in absorption was obtained from an association of the purified and detergent-solubilized proteins in micelles and was shown to be reversible with a half-time of 24 min by increasing the ionic strength with KCl. The observed magnetic CD change that resulted from complex formation of cytochrome P-450 and cytochrome $b_5$ in the reconstituted vesicles decreased within hours after addition of 0.3 M KCl. An interaction of high affinity between cytochrome $b_5$ and another form of cytochrome P-450 (26) has been shown in Triton X-100 micelles.

Cytochrome P-450 molecules were shown by EPR spectroscopy[1] to be equally distributed in terms of proteins/nm$^2$ on both inside and outside of the vesicles. This evidence was obtained by reconstituting spin-labeled cytochrome P-450 in vesicles, measuring the rate of reduction of the nitroxide group by ascorbic acid, and analyzing the biphasic kinetic data. The outer surface area of about 60% of the total contains 60% of the proteins. In a complete vesicle-reconstituted system consisting of reductase, cytochrome $b_5$, and cytochrome P-450, addition of KCl decreased metabolic activity immediately to 54% of the original value at low ionic strength. In the absence of cytochrome $b_5$, addition of KCl decreased the activity to 38–41% of the control value, which corresponds to the loss of activity of all cytochrome P-450 molecules in the outer monolayer. The higher remaining activity at high ionic strength in vesicles with cytochrome $b_5$ is numerically identical with the activity increase upon incorporation of cytochrome $b_5$ measured in low ionic strength buffer. The identity of these values suggests that direct electron flow from reductase to cytochrome P-450 is interrupted by high ionic strength whereas the second pathway through cytochrome $b_5$ is not affected on a time scale of minutes. The interaction of reductase and cytochrome P-450 seems to depend on the correct formation of an ionic bond for electron transfer. A similar electrostatic interaction might also exist between cytochrome P-450 and cytochrome $b_5$ because at high ionic strength the magnetic CD difference spectrum that resulted from mixing the two proteins disappeared within hours. The most simple mechanism of interaction of the three membrane proteins consistent with the above data would be reversible association reactions between the three possible pairs of different proteins that result in formation of dimers. Electron transfer from reductase to cytochrome P-450 or cytochrome $b_5$ or from cytochrome $b_5$ to cytochrome P-450 would occur during the lifetime of these dimers. The increase in the magnetic CD difference spectra as well as the alterations in metabolic activity (7, 27, 28) caused by addition of cytochrome $b_5$ may be explained either by formation of a ternary complex, reductase-cytochrome $b_5$-cytochrome P-450, or by a shift of the association equilibria between the three possible dimers and their monomeric forms.

*Acknowledgments*—We would like to thank Marie Bendix and Ruth Records for expert technical assistance, Betty Hampton for typing the manuscript, and Audrey Stevens for editorial help.

[1] B. Bösterling and J. R. Trudell, unpublished data.

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**Association of cytochrome b5 and cytochrome P-450 reductase with cytochrome P-450 in the membrane of reconstituted vesicles.**

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*J. Biol. Chem. 1982, 257:4783-4787.*