Physicochemical Characterization of CitrateSynthase
and Its Subunits*

JANG-YEN WU† AND JEN TSI YANG
From the Department of Biochemistry and Biophysics and Cardiovascular Research Institute, University of California Medical Center, San Francisco, California 94122

SUMMARY

Citrate synthase has a molecular weight of $1.0 \times 10^5$, as determined from sedimentation equilibrium and also from a combination of hydrodynamic properties. The sedimentation and diffusion coefficients were found to be 6.0 S and $5.8 \times 10^{-7} \text{ cm}^2 \text{s}^{-1} \text{ mac}^{-1}$, respectively, and the intrinsic viscosity was 3.95 ml g$^{-1}$. Optical rotatory dispersion showed a 233 m$^{-1}$ trough and a 108 m$^{-1}$ peak with $\Delta [\theta]_{222} = -7,150$ and $\Delta [\theta]_{198} = +28,500 \text{ deg cm}^2 \text{ mole}^{-1}$; the $b_0$ of the Moffitt equation was $-240$. Circular dichroism showed a double minimum at 222 and 210 m$^{-1}$ and a maximum at 198 m$^{-1}$ with $[\theta]_{222} = -20,000$, $[\theta]_{198} = -21,000$, and $[\theta]_{198} = +42,000 \text{ deg cm}^2 \text{ mole}^{-1}$. These findings indicate the presence of a moderate amount of $\alpha$-helical segments in the protein molecule.

Citrate synthase does not dissociate in the presence of 2 M KCl, dioxane, 8 M urea, $p$-chloromercuribenzoate, or $p$-mercaptoethanol, but it can be readily split into two physically indistinguishable subunits upon succinylation. The succinylated subunit was enzymatically inactive. Its sedimentation coefficient was one-half that of the native enzyme and its diffusion coefficient was reduced by about 10%.

The optical properties also suggested a helical content of about one-half of the native enzyme molecule. Citrate synthase can also dissociate into two subunits in 6 M guanidine hydrochloride, but the molecule becomes completely unordered.

The enzyme. Since the model of Monod, Wyman, and Changeux (3) for allosteric proteins requires the participation of subunits within the enzyme molecule, one of our main objectives was then the identification and characterization of these subunits if they were present. We first studied the size and conformation of native citrate synthase by a combination of hydrodynamic and optical measurements. Next, we attempted to dissociate the protein molecule into subunits with various treatments, and succeeded with succinylation and with 6 M guanidine hydrochloride. This was then followed by studying the size and conformation of the two physically indistinguishable subunits (succinylated).

EXPERIMENTAL PROCEDURE

Materials—Citrate synthase from pig heart was purchased from Boehringer Mannheim and also prepared in this laboratory by the method of Srere and Kosicki (4). The commercial sample was a crystalline suspension in 2.2 M ammonium sulfate (pH about 7). Its enzymatic activity was approximately 70 units per mg; the contaminants, based on the specific activity, were less than 0.1% aconitate and isocitrate dehydrogenase and less than 0.1% malate dehydrogenase. The enzyme was assayed at pH 8.2 with the method of Srere, Brazil, and Gonen (5).

The citrate synthase solution was prepared as follows. The protein suspensions were dialyzed against 0.1 M Tris buffer (pH 8.2) or 0.1 M phosphate buffer (pH 7.4) at 4° for at least 24 hours. (The dialysis tubings had been washed with 1% EDTA to remove any heavy metal ions, then with boiling distilled water, hot 5% sodium bicarbonate, and finally with ethanol.) The dialyzed solution was centrifuged at 12,000 rpm with a Sorvall RC 2-B centrifuge for 20 min to remove any precipitate. For chemical modifications, the enzyme suspensions were dialyzed against the buffer containing appropriate reagents.

Succinylation of citrate synthase was carried out according to the method of Habob, Cazelaix, and Slinger (6), with 12 moles of succinic anhydride for each mole of free amino group (assuming 10 moles/100 moles of lysine in the protein molecule). The pH of the reaction mixture was maintained at 8.2 by adding 1 M NaOH; the reaction proceeded at 4° for 3 hours and excess succinic anhydride was removed through dialysis against 0.1 M Tris buffer for 24 hours.

The concentration of the enzyme solution was determined by the Folin-Ciocalteu method and checked against a standard solution of crystalline bovine plasma albumin (7). For routine analysis, the concentration was also measured spectrophoto-
metrically on a Zeiss PM II spectrophotometer with $E_{1\text{cm}}^{1\%} = 15.0$ at 280 nm on the basis of Form-Ciocalteu method.

**Sedimentation and Diffusion**—All sedimentation experiments were made with a Spinco model E analytical ultracentrifuge. Sedimentation velocity at 20° was measured at 59,780 rpm for native protein and 66,100 rpm for succinylated protein, with the use of schlieren optics. Diffusion was run in a synthetic boundary double sector cell at 9,945 rpm at 20°. Sedimentation equilibrium was carried out at 4° or 20° in Yphantis’ cells (both six-channel rectangular (8) and eight-channel circular (9)) with the use of Rayleigh interference optics.

Diffusion coefficient, $D$, was calculated from the slope of a plot of $(A^2/H^2)$ against $t$ according to the equation:

$$D = (A^2/H^2)/4\pi t$$

where $A$ is the area under the diffusion peak in centimeters, $H$ is the maximum height of the peak in centimeters, and $t$ is the time in seconds (10). The area was calculated by trapezoidal integration. All areas were converted to zero time according to the formula $A_0 = A_x + A_y$, where $A_x$ and $A_y$ refer to the boundary position at time, $t$, and zero, respectively. The mean value of $A_0$, $\bar{A}_0$, was calculated and then a smoothed value of $A$, was obtained from $A_x = \bar{A}_0 (x_0/x_0^2)$ and used in Equation 1. Both the sedimentation and diffusion coefficients at infinite dilution, $s$ and $D'$, were obtained by linear extrapolation of the $s$-C and $D$-C plots to zero concentration.

**Viscometry**—Viscosity was measured in an Ubbelhode type viscometer with solvent flow time of more than 800 sec at 20° ± 0.1°. The kinematic viscosity, $[\eta]$, thus determined, was converted into intrinsic viscosity, $[\eta]$, according to the suggestion of Tanford (11):

$$[\eta] = 10 - (t_1 - t_2)/(4C)$$

and

$$[\eta] = [\eta] + (1 - \eta_0)/100\rho_0$$

where $\rho_0$ is the density of the solvent.

**Molecular Weight Determination and Partial Specific Volume**—The apparent molecular weight, $M^{app}$, of the protein at any finite concentration was calculated from the standard equation:

$$M^{app} = 2RT(d\ln C/dr^2)/\omega^2(1 - \eta_0)$$

where $\omega$ is the angular velocity and $C$ is the concentration (expressed in terms of the Rayleigh fringe displacements). The plot of $(1/M^{app})$ against $C$ was linear and its intercept at zero concentration gave the reciprocal of the weight average molecular weight of the protein.

Edelstein and Schachman (12) proposed that the molecular weight and partial specific volume, $\bar{v}$, of a macromolecule can be determined simultaneously from sedimentation equilibrium in both $H_2O$ and $D_2O$. This method is based on the changes produced in the concentration distribution at equilibrium due to increase in the density of solution when $D_2O$ (or $D_4PO$) replaces $H_2O$ as the solvent. Thus, $\bar{v}$ can be calculated according to the equation:

$$\bar{v} = k - (d\ln C/dr^2)_H / (d\ln C/dr^2)_D$$

with a pycnometer. A $k$ value of 1.0155 was reported for bovine plasma albumin and found to be relatively constant for all proteins (13). With $\bar{v}$ determined we can calculate $M^{app}$ from the standard equation for sedimentation equilibrium (Equation 3).

The molecular weight of the protein can also be determined from the sedimentation and diffusion coefficients, $s$ and $D'$, with the Svedberg equation. For an additional check, we can estimate the molecular weight of the native protein from the Scharaga-Mandelkern equation:

$$M^{app} = N\bar{v}^2(1 + \eta_0/\rho)(1 - \eta_0/\rho)$$

by using a reasonable value for $\beta$ (see Reference 14).

**ORD** and **CD**—ORD was measured with a Cary model 60 recording spectropolarimeter and CD with a Jasco ORD/UV-5 with a CD attachment, both under a constant nitrogen flush. Specially designed thermostatable cell holders and jackets were installed in both instruments. The data were expressed in terms of reduced mean residue rotation, $[\eta]'$, and mean residue ellipticity, $[\theta]$ (without reflective index correction), respectively (15). The rotation data in the visible region were analyzed with the Moffitt equation (16):

$$[\eta]' = -100([\eta]'_H2O + 2,000)/13,000$$

or

$$[\theta]_H2O = 100(4,800 - [\theta]_D2O)/45,400$$

or

$$[\theta]_H2O = 100([\theta]_D2O + 2,200)/36,200$$

Similar expressions can be deduced from the 198 Hz peak of ORD and 191 Hz maximum of CD.

**Amino Acid Analysis**—The amino acid composition of citrate synthase was analyzed by the method of Moore and Stein (17). One milliliter of 0.1% protein solution was mixed with 1 ml of concentrated HCl in the ignition tube. The mixture was frozen in Dry Ice and degassed exhaustively under vacuum. After sealing, the tube was heated at 110° for 24, 48, or 72 hours. The hydrolyzed products were then transferred to a 50-ml round bottom flask and evaporated to dryness. The dried hydrolyzates were redissolved in 2 ml of citrate buffer (pH 2.2). Aliquots of this solution were then analyzed in an automatic amino acid analyzer and the results, after heating for 24, 48, or 72 hours, were extrapolated to zero time. These analyses were carried out in the laboratory of Professor F. H. Carpenter of the University of California at Berkeley.

Tryptophan was determined spectrophotometrically according to the method of Edelhoch (18), except that 0.5 mg of EDTA per ml was included in the protein solution. The half-cystine and $\gamma$-amino groups would be alkylated with 5% iodoacetic acid.

$^1$ The abbreviations used are: ORD, optical rotatory dispersion; CD, circular dichroism.

$^2$ W. F. Cleland, J. T. Yang, data to be published.
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FIG. 1. Sedimentation equilibrium plot: Rayleigh fringe displacement (in microns) plotted against the square of the distance (in centimeters) from the center of rotation. Concentrations: 0.3 mg per ml. Picture taken 45 hours after the rotor reached 27,690 rpm at 4°.

TABLE I
Molecular weight of citrate synthase by sedimentation equilibrium
In 0.1 M Tris buffer, pH 8.2, at 4° and 27,690 rpm for 45 hours.

| Protein concentration (mg/ml) | M^app |
|------------------------------|-------|
| 0.30                         | 100,000 |
| 0.45                         | 100,000 |
| 0.60                         | 100,000 |
| 0.90                         | 99,000 |

methionine contents were determined by the method of Hirs with performic acid oxidation (19). The sulfhydryl groups were also estimated by titrating the protein solution in 6 M guanidine hydrochloride with Ellman's reagent (20); the denaturing agent was used to effect the unfolding of the protein molecule.

RESULTS

Molecular Weight of Native Citrate Synthase—We determined the molecular weight of the protein by sedimentation equilibrium. Fig. 1 plots the logarithm of concentration, C, in terms of the Rayleigh fringe displacements, µ in microns, against the square of the distance, r in centimeters, from the center of rotation in both H_2O and D_2O. We then calculate the β in Equation 4 from the slopes in the figure. Our density measurements indicated a 93% D_2O in our experiment, which gave k = 1.0144 through interpolation (k = 1.0155 in a 100% D_2O solution). This, in turn, gave a β of 0.733 for citrate synthase. From Equation 3, we then determined the M^app, which was virtually concentration-independent in the range of concentrations used (Table I). Thus, we obtained a weight average molecular weight of 1.0 × 10^5 for citrate synthase at infinite dilution.

Hydrodynamic Properties of Citrate Synthase—Table II lists all of the pertinent results of the hydrodynamic and optical properties of citrate synthase and its subunits (see below). In all cases the sedimentation velocity patterns showed a single symmetrical peak suggesting the homogeneity of the preparations (figures not shown). Fig. 2 illustrates a representative plot for the diffusion runs. From the slope we can calculate the diffusion coefficient, D, according to Equation 1 (see “Experimental Procedure”). The diffusion coefficient, D_o, was then determined by extrapolating D to zero concentration.

The molecular weight of citrate synthase as determined from the combination of s^0 and D (the Svedberg equation) is very close to that obtained directly from sedimentation equilibrium (Table II). This is also true for the estimated molecular weight based on the Scheraga-Mandelkern equation (Equation 5). In this case we assumed β = 2.1 × 10^5 and used [η] = 0.0385 dl per g. The β value was so chosen because of the low intrinsic viscosity of the native protein, suggesting a very compact globular molecule.
Optical Properties of Citrate Synthase—Figs. 3 and 4 show the ORD and Fig. 5 shows the CD of citrate synthase. All pertinent data are included in Table II. The characteristic 233 nm trough and 198 nm peak of ORD (Fig. 3) are indicative of the presence of $\alpha$-helical segments in the protein molecule as are the typical double minimum at 222 and 210 nm and the maximum at 191 nm from the CD spectrum (Fig. 5). The ORD in the visible region obeys the Moffitt equation (16); its negative $\beta_0$ again suggests the presence of $\alpha$-helices.

Amino Acid Composition of Citrate Synthase—Table III lists the amino acid composition of the enzyme. The contents of asparagine and glutamine were not determined in our analysis. Our estimate of the free sulfhydryl groups was about eight, suggesting that citrate synthase contains at most one cystine group and very likely none at all.

Succinylation of Citrate Synthase: Number of Subunits—One of our main objectives was an attempt to split this regulatory enzyme into its subunits, if any, by various treatments. Therefore, we measured the sedimentation coefficient and also the optical properties of citrate synthase after treatment with the following reagents: salt of high concentration, organic solvents, urea, guanidine hydrochloride, anionic detergent, $p$-chloromercuribenzoate, $\beta$-mercaptoethanol, and, finally, succinic anhydride. Our first criterion was to detect any marked change in the sedimentation coefficient of citrate synthase in a dilute solution. In all cases we observed a single sedimentation peak. Judging from the $s_{20, w}$ values in Table IV, only treatment with 5 m guanidine hydrochloride, 1% sodium dodecyl sulfate, or succinylation might have split citrate synthase into two or more ultracentrifugally indistinguishable subunits. The use of salt as high as 2 m KCl did not significantly change either the hydrodynamic or optical properties of citrate synthase (Table IV) and...
the enzyme was fully active in a standard assay, nor did treat-
ment with dioxane, p-chloromercuribenzoate, or β-mercapto-
ethanol seem to dissociate the protein into subunits or to effect 
its optical properties very much (Table IV). With p-chloro-
mercuribenzoate, the protein solution became turbid and precise 
measurements of its optical properties were very difficult to 
obtain. The enzyme was still partially active in the presence of 
these organic reagents.

We concentrated our effort on the modification of citrate syn-
thase by succinylation. Sedimentation velocity experiments of 
succinylated citrate synthase showed a single symmetrical peak. 
The corresponding $s$ is about one-half that of the native protein, 
whereas the diffusion coefficient, $D$, only alters slightly upon 
succinylation (Table II). These observations suggest that the 
protein molecule was split into two physically indistinguishable 
subunits after treatment with succinic anhydride. This is fur-
ther substantiated by the molecular weight determination which 
gave a value of about 54,000 from sedimentation equilibrium at 
one finite concentration (Fig. 6, Curve C) and 55,000 from the 
combination of $s$ and $D$ values. Although the number of suc-
cinyl groups covalently bound to the protein molecule was not 
analyzed, the $\varepsilon$-NH$_2$ groups of the lysine residues (see Table III) 
were expected to be completely succinylated in the presence of 
excess succinic anhydride. The fully succinylated protein will 
increase its molecular weight by about 4,600 because of the 
succinyl groups which have a residue molecular weight of 100. 
Thus, each subunit should have a molecular weight of 52,000 to 
53,000, which is in accord with the values listed in Table II.

The succinylated citrate synthase was no longer enzymatically 
active. Its optical properties were also altered; the ORD still

**Table III**

| Amino acid composition of citrate synthase |
|------------------------------------------|
| A | B |
|---|---|
| Lysine | 46 | 5.34 |
| Histidine | 25 | 2.90 |
| Arginine | 38 | 4.40 |
| Aspartic acid$^a$ | 80 | 9.28 |
| Threonine | 44 | 5.10 |
| Serine | 50 | 5.80 |
| Glutamic acid$^a$ | 84 | 9.74 |
| Proline | 44 | 5.10 |
| Glycine | 65 | 7.54 |
| Alanine | 65 | 7.54 |
| Half-cystine | 10 | 1.16 |
| Valine | 54 | 6.26 |
| Methionine | 28 | 3.23 |
| Isoleucine | 36 | 4.18 |
| Leucine | 110 | 12.70 |
| Tyrosine | 32 | 3.71 |
| Phenylalanine | 25 | 2.90 |
| Tryptophan | 26 | 3.02 |
| **Total** | **862** | **100** |

$^a$ The figures included both free and amidated residues of these amino acids.

**Table IV**

| Hydrodynamic and optical properties of citrate synthase under various conditions |
|-----------------------------------------------|
| Reagents | $S$ (mg/ml) | $[\pi]^2$ | $[\kappa]^2$ | $\theta_{122}$ | $\theta_{115}$ |
| 1. 0.1 M Tris buffer, pH 8.2. | 5.6$^a$ | 6 | -7,150 | +28,500 | -20,000 | -21,000 |
| 2. 2 M KCl | 4.3 | 5.6 | -6,100 | +31,000 | -15,000 | -19,000 |
| 3. 8 M urea | 4.8 | 6.5 | -2,800 | -3,000 |
| 4. 6 M guanidine HCl | 2.2 | 7.8 |
| 5. 10% (v/v) dioxane | 5.6 | 8.3 | -5,600 | -16,700 | -18,100 |
| 6. 20 M guanidine HCl. | 6.5 | 7.5 |
| 7. 0.1 M β-mercaptoethanol | 3.3 | 7.5 | -15,000 |
| 8. 1% (w/v) sodium dodecyl sulfate | 2.9 | 8.0 | -4,700 | -9,700 | -19,000 |
| 9. Succinic anhydride$^c$ | 2.0 | 6 | -4,600 |

$^a$ Read from the (1/s) - C plot.

$^c$ CMB, p-chloromercuribenzoate.

$^c$ See test for detail of succinylation.
retained the 233 nm trough (Fig. 3, Curve 2) and the CD retained the double minimum (Fig. 5, Curve 2), but their magnitudes were reduced (Table II), suggesting a decrease in the helical content after succinylation. The same was true for the rotations in the visible region, where the levorotations of the protein increased upon succinylation and the -\( b_0 \) of the Moffitt equation became small as compared with that of the native protein. To summarize: both the hydrodynamic and optical properties of citrate synthase are altered significantly when the protein molecule is subunits (succinylated) may differ from that of the native protein

Subunits in Presence of Denaturing Agent—Since the sedimentation coefficient of citrate synthase was markedly reduced in guanidine hydrochloride solution (Table II), we determined the molecular weight of this protein in 6 M guanidine hydrochloride with and without 0.1 M \( \beta \)-mercaptoethanol by Yphantis’ high speed sedimentation equilibrium method (Fig. 6, Curves A and B). Assuming that the partial specific volume of the protein remains the same as that of the native protein, we obtained a weight average molecular weight of 49,000 in both cases. This suggests that citrate synthase can also be dissociated into two physically indistinguishable subunits by this denaturing agent.

Furthermore, since the presence of a reducing agent does not affect the results, such a dissociation does not involve the cleavage of the disulfide bond, if any, between the subunits. Rather, it is caused by the disruption of noncovalent interactions that hold the two subunits together. This is also consistent with the observation that succinylation of citrate synthase only splits the molecule into two subunits.

Sodium dodecyl sulfate also seems promising for the dissociation of citrate synthase (Table II). However, experimental difficulties made accurate molecular weight determination difficult.

### Discussion

The molecular weight of citrate synthase as reported in the literature is rather unsettled. Srere and Kosicki (4) found the enzyme from pig heart to have a sedimentation and diffusion coefficient of 5.0 S and 7.5 \( \times 10^{-7} \) cm² per sec, which gave a molecular weight of 56,000, assuming \( \theta = 0.70 \) ml per g. Later, Srere et al. (5) reported an \( \theta \) value of 4.5 S for this enzyme from moth, 6.2 S from pigeon, and 6.1 S from pig heart. More recently, Srere (21) revised the molecular weight to 67,000 based on sedimentation and diffusion measurements and to 80,000 using the Archibald’s approach to equilibrium method. Srere and Senkin (2) also reported a value of 55,000 for citrate synthase from pig heart. Our sedimentation coefficient of 6.0 S agrees with the recent value reported by Srere and associates, but our diffusion coefficient of 5.8 \( \times 10^{-7} \) cm² per sec is much smaller than their 7.5 \( \times 10^{-7} \) cm² per sec. The discrepancy between our molecular weight results and theirs could be partly attributed to the difference in the partial specific volume used. If \( \theta = 0.733 \) ml per g instead of 0.70 ml per g is used, their molecular weight results would have been raised by about 12%. For instance, their value of 55,000 would become 95,000, which is close to our value of 1.0 \( \times 10^4 \).

The low intrinsic viscosity of native citrate synthase clearly indicates that this globular protein is very compact and not highly asymmetrical. Calculation of the frictional ratio, \( f/s_0 \), from the sedimentation and diffusion coefficients (14) leads to the same conclusion. On the other hand, analysis of the hydrodynamic measurements of the succinylated subunits suggests that each subunit is more asymmetrical than the native protein. Consequently, the two subunits could be associated side by side, thus reducing the asymmetry of the native protein molecule. Since the optical properties indicate some significant changes in conformation upon succinylation, the shape of the dissociated subunits (succinylated) may differ from that of the native protein and any unfolding of the molecule will further contribute to an increase in the asymmetry of the subunits.

The Cotton effects of citrate synthase and its succinylated subunits (Figs. 3 to 5) display the extrema that are characteristic of \( \alpha \)-helix. The ORD in the visible region shows a levorotation of the native protein less than that expected of a protein molecule containing a moderate amount of the helix. This is more obvious in comparing the Moffitt parameters of citrate synthase with other globular proteins. Our results show a positive \( a_0 \) of 30 as contrasted with a negative one that is common for most globular proteins having a \( b_0 \) in the range of our observation (-240). (Srere (22) reported \( a_0 = -4 \) and \( b_0 = -230 \).) One other known exception is glutamate dehydrogenase, which has an \( a_0 \) of +120 and a \( b_0 \) of -200 to -270 (23). The origin of this “anomaly” is not clear at present, but it could be due to the presence of other secondary structures such as the \( \beta \)-form, which may alter the \( a_0 \) but not the \( b_0 \) (24). Since the contribution of the \( \beta \)-form to the optical activity of a protein molecule is overshadowed when \( \alpha \)-helix and \( \beta \)-form coexist, there is presently no satisfactory method of analysis that will overcome this problem, numerous attempts described in the literature notwithstanding. Succinylation of citrate synthase does reverse \( a_0 \) from positive to negative and reduce the magnitude of \( b_0 \) (Table II), suggesting some significant change in the secondary and even tertiary structures of the protein molecule.

Using experimental values listed in Table II, we estimated for native citrate synthase a helical content of about 40% from the 233 nm trough method (Equation 7a) as well as the \( b_0 \) method (see Equation 6). On the other hand, the CD data gave an estimate of about 55% and 50% from the 222 and 210 nm minimum (Equations 7b and 7c), respectively. Of course, such analysis is subject to several uncertainties (for a review, see Reference 24), and it will be rather premature to attempt to resolve these discrepancies at this stage of development. Nevertheless, our results indicate that native citrate synthase does contain a moderate amount of \( \alpha \)-helix. ORD and CD are especially powerful in detecting any conformational changes that accompany physical and chemical treatments. Thus, we can reasonably conclude from the data in Table II that succinylated citrate synthase subunits contain relatively less amount of \( \alpha \)-helix than the native enzyme, although succinylation does not destroy the secondary structure of the protein molecule completely. On the other hand, citrate synthase becomes unordered in 6 M guanidine hydrochloride or 8 M urea.

Succinylation has been used for the dissociation of proteins such as methemerythrin and oxyhemerythrin (25) and catalase (26). Habecb et al. (6) reported that succinic anhydride attacked specifically and exhaustively the free amino groups in a protein molecule. Based on our amino acid analysis (Table III), citrate synthase contains about 46 lysine residues in a molecule of about 600 amino acid residues. Thus, succinylation results in a conversion of about 23 positive charges into negative ones for each of the...
two subunits—a net change of about 46 charges/430 amino acid residues. Clearly, electrostatic repulsion could play an important role in the dissociation process, which is also responsible for the change in shape and conformation of the protein molecule. Without knowledge of the charge distribution, however, it is not possible to distinguish the following two possibilities: Some of the free amino groups could have been located in a critical position that holds the two subunits together. For instance, the positively charged lysine residues on one subunit might be salt-linked to the negatively charged carboxylate groups on the other. Alternately, some positively and negatively charged groups could be arranged in juxtaposition to the surface where the two subunits contact. Succinylation would increase the net negative charges in these positions, which results in an electrostatic repulsion among the charged groups between the two subunits, thus making their association unstable.

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REFERENCES
1. Shephard, D., and Garland, P. B., Biochem. Biophys. Res. Commun., 22, 89 (1966).
2. Wieland, O., and Weiss, L., Biochem. Biophys. Res. Commun., 13, 26 (1963).
3. Monod, J., Wyman, J., and Changeux, J., J. Mol. Biol., 12, 58 (1965).
4. Sheere, F. A., and Kosicki, G. W., J. Biol. Chem., 236, 2557 (1961).
5. Sheere, F. A., Brazil, H., and Guenn, L., Acta Chem. Scand., 17, 8129 (1963).
6. Habeeb, A. F. S. A., Cassidy, H. G., and Singer, S. J., Biochem. Biophys. Acta, 29, 857 (1958).
7. Folin, O., and Ciocalteu, V., J. Biol. Chem., 73, 627 (1927).
8. Yphantis, D. E., Biochim. Biophys. Acta, 8, 41 (1959).
9. Yphantis, D. E., Ann. N. Y. Acad. Sci., 88, 889 (1960).
10. Baldwin, T. L., Biochem. J., 65, 503 (1967).
11. Tanford, C., J. Phys. Chem., 69, 708 (1955).
12. Edelstein, S. J., and Schachman, H. K., J. Biol. Chem., 242, 306 (1967).
13. Hvidt, A., and Neilsen, S. O., Advan. Protein Chem., 21, 287 (1966).
14. Yang, J. T., Advan. Protein Chem., 16, 323 (1961).
15. Yang, J. T., in P. Alexander and H. P. Lundgren (Editors), A laboratory manual of analytical methods in protein chemistry, Vol. 5, Chap. 2, Pergamon Press, Oxford, 1969.
16. Moffett, W., and Yang, J. T., Proc. Nat. Acad. Sci. U. S. A., 42, 596 (1956).
17. Moore, S., and Stein, W., in S. P. Colowick and N. O. Kaplan (Editors), Methods in enzymology, Vol. VI, Academic Press, New York, 1963, p. 619.
18. Edelhoch, H., Biochemistry, 6, 1948 (1967).
19. Hirs, C. H. W., J. Biol. Chem., 219, 611 (1956).
20. Edman, G. L., Arch. Biochem. Biophys., 70, 70 (1959).
21. Sheere, F. A., Biochim. Biophys. Acta, 106, 445 (1965).
22. Simms, P. A., J. Biol. Chem., 241, 2157 (1966).
23. Magar, M. E., Ph.D. Thesis, University of California San Francisco Medical Center, 1966.
24. Yang, J. T., in G. D. Fasman (Editor), Poly α-amino acids, Chap. 6, Marcel Dekker, New York, 1967.
25. Keresztes-Nagy, S., and Klotz, I. M., Biochemistry, 2, 923 (1963).
26. Sund, H., Weber, K., and Molbert, E., Eur. J. Biochem., 1, 400 (1967).
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