Active Enzyme Sedimentation, Sedimentation Velocity, and Sedimentation Equilibrium Studies of Succinyl-CoA Synthetases of Porcine Heart and *Escherichia coli*†

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ABSTRACT: Succinyl-CoA synthetases from *Escherichia coli* and porcine heart muscle have been viewed as prototypes of two classes of the enzyme. The bacterial enzyme has been reported to be an $\alpha_2\beta_2$ tetramer, with many suggestions in the literature for cooperative interactions between active sites that may contribute to its catalytic efficacy. In contrast, gel filtration experiments of others have indicated that the heart enzyme is a simple $\alpha\beta$ dimer, with no evidence of dimerization or interaction between like sites. All previous estimates of molecular size of these enzymes have been carried out at concentrations that are much higher than those that are used during activity measurements. The present study was carried out to confirm the differences in the quaternary structures of these two species of succinyl-CoA synthetase and to extend our knowledge of these structures to very low concentrations to enable correlation of their subunit structures with their catalytic properties. Conventional sedimentation velocity centrifugation with both enzymes indicates behavior typical of noninteracting globular proteins with no evidence of size heterogeneity. The sedimentation coefficients at infinite dilution ($s^\circ_{20,w}$) have been determined to be 7.04 S and 4.55 S for the *E. coli* and porcine heart enzymes, respectively. Sedimentation velocity measurements have been extended to very low enzyme concentrations (typical of those used in activity measurements) by active enzyme centrifugation experiments, in which we have determined the rate of sedimentation of a zone of active enzyme through a chromogenic substrate solution. These measurements confirm that the differences in sedimentation coefficients extend to assay concentration and give no indication of dissociation of the tetrameric *E. coli* enzyme nor association of the dimeric porcine heart enzyme. Sedimentation equilibrium experiments have been performed for both enzymes over a wide range of loading concentrations. Consistent with observations made with previous sedimentation equilibrium runs, *E. coli* succinyl-CoA synthetase behaves anomalously at low concentrations (<2 mg/mL), indicating the presence of one or more components that are less than the tetramer in size. The sedimentation equilibrium data exclude a simple dissociation–equilibrium system, however, and the active enzyme centrifugation results with the same enzyme show that the smaller components, whatever their nature, have no significant activity. At higher concentrations of *E. coli* succinyl-CoA synthetase where the anomalous behavior is not observed, the apparent molecular weight is concentration-independent and was determined to be 139 000, in good agreement with the value of 142 000 that has been calculated from its amino acid sequence and an $\alpha_2\beta_2$ structure [Buck, D., Spencer, M. E., & Guest, J. R. (1985) *Biochemistry 24*, 6245–6252]. Thus, appropriate correlation of molecular weight to sedimentation coefficient confirms that the active species is the tetramer. The porcine heart enzyme, in contrast, has a M_r of 78 000, with no detectable formation of larger aggregates even at concentrations in excess of 10 mg/mL, and reduced tendency to form anomalous smaller components. Thus, correlation of all of our sedimentation data obtained over a wide concentration range (ca. 1 μg/mL to 10 mg/mL) establishes that the active form of *E. coli* succinyl-CoA synthetase is a nondissociating $\alpha_2\beta_2$ tetramer while the porcine heart species of enzyme is a nonassociating $\alpha\beta$ dimer.

Succinyl-CoA synthetase catalyzes the "substrate-level" phosphorylation step of the tricarboxylic acid cycle:

$$\text{succinyl-CoA} + \text{NDP} + \text{P}_i \rightleftarrows \text{succinate} + \text{NTP} + \text{CoA}$$

While its activity is therefore virtually ubiquitous, succinyl-CoA synthetases from two sources in particular, namely, *Escherichia coli* and porcine heart mitochondria, have been the subjects of almost all investigations of structure and function. For many years, these have been regarded as representative of two distinct types of SCS.[1] For example, the enzyme from *E. coli* prefers adenine nucleotides as substrates, while heart SCS is specific for guanine nucleotides [for a review, see Bridger (1974)]. Differences have been thought to extend to the molecular sizes and subunit structures of the two enzyme types. The enzyme from *E. coli* and other Gran-negative bacteria has been reported to have a molecular weight of approximately 140 000 (Bridger, 1974; Krebs & Bridger, 1974; Weitzman & Kinghorn, 1978) and to be an $\alpha_2\beta_2$ tetramer. Earlier estimates of the sizes of the α and β subunits of the *E. coli* enzymes (Bridger, 1971) have been confirmed by the recent report by Buck et al. (1985), who have deduced the primary sequences of the α and β subunits from

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[1] Abbreviations: SCS, succinyl-CoA synthetase; AEC, active enzyme centrifugation; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid.

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the nucleotide sequences of their respective genes and have calculated molecular weights of 29 644 for α and 41 390 for β. The porcine heart enzyme contains these two subunit types (Brownie & Bridger, 1972), but its gel filtration behavior suggests that it is an $\alpha\beta$ dimer, with a molecular weight near 77 000 (Baccanari & Cha, 1973; Brownie & Bridger, 1972; Bild et al., 1980).

Many recent studies of this enzyme have involved attempts to gain an understanding of the functional rationale for its subunit structure. For both the bacterial and the mammalian enzymes, it is clear that the α subunit contains the histidine residue that is transiently phosphorylated during the course of the reaction, together with the binding site for nucleoside triphosphate, while the β subunit contains the binding sites for succinate and coenzyme A (Bridger, 1974; Pearson & Bridger, 1975; Collier & Nishimura, 1978). This distribution of binding sites for substrates thus accounts for the presence of two types of subunits in SCS. More equivocal, however, is the rationale for the apparent dimer of dimers represented by the $\alpha_2\beta_2$ structure proposed for *E. coli* SCS. On the basis of the kinetics of exchange of ^{18}O exchange between succinate and phosphate (Bild et al., 1980), properties of hybrid enzyme preparations (Wolodko et al., 1981), ^{31}P NMR studies (Vogel & Bridger, 1982; 1983), and the marked stimulatory effects of ATP on the discharge of thiophosphate from the enzyme in the presence of succinate and CoA (Wolodko et al., 1983), it has been proposed that the two $\alpha\beta$ halves of SCS function in alternating fashion, with exergonic phosphorylation of one site promoting more difficult partial reactions at the neighboring active center. However, Nishimura and his colleagues have called some of these conclusions into question. For example, they have shown that technical limitations may cloud the interpretation of the ^{18}O exchange kinetic studies (Nishimura & Mitchell, 1984). Moreover, they have shown that the porcine heart enzyme, which has been generally considered to be a simple $\alpha\beta$ dimer, has equivalent properties to those of *E. coli* SCS with respect to the stimulatory effects of nucleoside triphosphates on the rate of its dethiophosphorylation (Nishimura & Mitchell, 1985). Clearly, then, comparisons of the properties of the bacterial and mammalian enzymes would be helpful in providing evaluation of the functional significance of tetrameric or dimeric subunit assembly in catalysis by SCS, provided that the long-standing proposals for their respective quaternary structures are correct.

These considerations have prompted this study. Here, we report detailed analysis of the physicochemical properties of SCS from both sources, confirming that the *E. coli* enzyme is $\alpha_2\beta_2$ and that the porcine heart enzyme is $\alpha\beta$. Significantly, these differences in molecular size extend to very low concentration ranges, as we deduce from measurements of the sedimentation rate of active enzyme at assay concentrations.

Experimental Procedures

Enzymes. Succinyl-CoA synthetase was isolated from both *E. coli* (Crooks strain grown on a phosphate-buffered succinate-based medium) and porcine heart muscle. The *E. coli* enzyme was purified from a thawed cell suspension essentially as described by Leitzmann et al. (1970). Significant modifications to this preparative procedure included the elimination of both the heat treatment step and the chromatography on hydroxylapatite and the inclusion of a final purification step involving affinity chromatography on Blue Sepharose CL-6B [Pharmacia (Canada) Ltd.]. The porcine enzyme was purified from fresh heart muscle by modifications to the method developed by Cha et al. (1967). Preparations were carried out in a single process starting with 15–20 hearts. Further important deviations from their procedure were as follows: extraction with water instead of buffer, elimination of all calcium phosphate gel chromatography, and final purification by affinity chromatography on Affi-Gel Blue (Bio-Rad Laboratories). Only potassium phosphate buffers were used throughout the purification procedures, since these were found to stabilize both enzymes and maintain high enzymatic activity. The enzymes were stored at 4 °C as suspensions in concentrated ammonium sulfate solutions. Protein concentrations were determined spectrophotometrically at 280 nm with values of $E^{1\%}_{1cm}$ equal to 5.0 for *E. coli* SCS (Krebs & Bridger, 1974) and 3.5 for porcine heart SCS (Maurakami & Nishimura, 1974). Enzymatic activity was assayed by the direct spectrophotometric method (Bridger et al., 1969) based on the increase in A_{232} accompanying succinyl-CoA formation. ATP was used as substrate with *E. coli* SCS, and GTP was used for porcine heart SCS.

Densities and Partial Specific Volumes. Since the partial specific volumes of proteins are known to vary significantly with temperature (Kay, 1959), it was important to determine this value for the two species of succinyl-CoA synthetase at the temperature to be used for our ultracentrifuge runs (5 °C). Densities of buffers and protein solutions were determined with an Anton Parr density measuring cell (DMA 601) and density meter (DMA 60). Measurements with the crystal oscillator were calibrated with dried air (density corrected for station barometric pressure) and distilled water (rendered dust free by Millipore filtration). The temperature was controlled at 5.00 ± 0.01 °C with a Lauda RM6 circulator. Enzyme solutions were dialyzed for a minimum of 48 h at 4 °C with three changes of the dialyzate buffer, followed by centrifugation for removal of particulate matter. The final dialyzate was used to measure the density of the buffer. The resultant densities of the protein solutions as determined from the measured frequencies were plotted against the corresponding concentrations; these data appeared linear and were fitted with a straight line by the method of least squares. Within the error of the plot, the density values obtained by extrapolation to zero protein concentration with both enzyme systems were identical with that measured for the dialyzate buffer alone. An apparent partial specific volume was calculated for each enzyme from the slope term as outlined by Van Holde (1975), based on Casassa–Eisenberg theory for dialyzed solutions. The density of the dialyzate buffer, 50 mM potassium phosphate–0.2 mM EDTA, pH 7.4 at 5.0 °C, was 1.00723 ± 0.00005 g/cm^3 (15 measurements), and values for the partial specific volume of *E. coli* and porcine heart succinyl-CoA synthetase in this buffer at 5 °C were found to be 0.759 ± 0.006 and 0.738 ± 0.009 cm^3/g, respectively.[2]

Ultracentrifugation. The sedimentation velocity and equilibrium studies were carried out with Beckman Spinco Model E analytical centrifuges each equipped with RTIC and electronic speed control units and, where appropriate, with a schlieren or a Rayleigh interference optical system or with a monochromator and photoelectric scanner with multiplexer. Titanium AnH or AnF rotors with newly calibrated thermistors were used throughout. The ultracentrifuge cells were loaded by means of glass syringes and Teflon needles to avoid introduction of heavy metals. All runs were performed at 5 °C.

Conventional sedimentation velocity experiments were conducted at speeds of 52 000 or 60 000 rpm. For protein

[2] These values compare with partial specific volumes at 25 °C of 0.747 and 0.741 cm^3/g that may be calculated from the amino acid compositions of *E. coli* and porcine heart succinyl-CoA synthetases, respectively.

concentrations greater than 2 mg/mL, observations were made in 12-mm path-length Kel-F-coated centerpieces with quartz windows and the schlieren optical system. The maximum ordinate of the symmetrical schlieren peak was taken as the boundary position in subsequent measurements of the photographs. For lower protein concentrations, 12-mm path-length charcoal-filled Epon double-sector centerpieces with sapphire windows were used, and the boundary was monitored at 280 or 235 nm with the monochromator and autoscanner. Sedimentation velocities were determined from the measured midpoints (curve inflection) of the scanner patterns and also from the equivalent boundary position $\bar{r}$ calculated as described by Teller (1973).

Active enzyme centrifugation (AEC) was carried out on the basis of the methodology developed by Cohen and Mire (1971). The catalytic activity of the enzyme was monitored during centrifugation by measuring the production of the free thiol of coenzyme A from succinyl-CoA by its reaction with DTNB, on the basis of an assay procedure developed by Leitzmann et al. (1970). Generation of the free mercaptide ion is thus followed spectrophotometrically at 412 nm. The rotor speed was 60 000 rpm for all AEC runs. The synthetic band of enzyme was formed in 12-mm path-length, Vinograd type I, charcoal-filled Epon, double-sector centerpieces with sapphire windows. A 10-μL volume of the given enzyme at a chosen concentration in 10-fold diluted dialyzate buffer was layered onto 300 μL of the appropriate DTNB-containing assay medium.[3] The assay media were prepared freshly for each run and consisted of 0.5 mM DTNB, 0.5 mM ADP (for *E. coli* SCS) or 0.5 mM GDP (for porcine heart SCS), 10 mM $MgCl_2$, and 0.48 succinyl-CoA in 50 mM potassium phosphate–0.2 mM EDTA, pH 7.4. We have confirmed the finding of Leitzmann et al. (1970) that activities measured in this medium are equivalent to those assayed by our conventional direct spectrophotometric method, indicating that the presence of low concentrations of DTNB does not affect the enzyme. Prior to the centrifuge run, the catalytic activity of the enzyme solutions was determined spectrophotometrically under equivalent assay conditions. On the basis of the measured activity, the amount of enzyme in the 10 μL to be introduced to the ultracentrifuge cell was adjusted to give absorbance changes on the scanner records in the range of 0.4–1, at least 7-fold below the change expected for completion of the reaction. Sedimentation velocity of the active enzymes was determined in three ways: midpoint (inflection) measurements, equivalent boundary position calculations (Teller, 1973), and difference curve constructions (Cohen & Mire, 1971). Subsequently, apparent sedimentation coefficients were obtained from linear least squares fit of ln radius vs. time plots and were corrected to standard conditions (Schachman, 1959) with values for viscosity from tables and experimentally determined solvent densities.

Low-speed sedimentation equilibrium experiments were conducted to determine the molecular weight distribution of the two enzymes under a variety of speeds and initial loading concentrations. Charcoal-filled Epon double-sector centerpieces, 12-mm path length, and sapphire windows were used with the Rayleigh interference optical system for protein concentrations greater than 1 mg/mL and with the monochromator and autoscanner for lower concentrations. Sedimentation equilibrium data were processed with the aid of computer programs written in this laboratory on the basis of methods described by Teller (1973). Apparent weight-average molecular weights were obtained at point concentrations by fitting ln C vs. r^2 data with a sliding least-squares line through each set of five successive points.

[3] By using less dense buffer for the sample, as described, we found it unnecessary to include a stabilizing density gradient generated by the presence of sucrose or heavy water as employed by others. Under our experimental conditions there was no evidence of enzyme sinking as indicated by the linear ln r vs. time plots (see Figure 2).

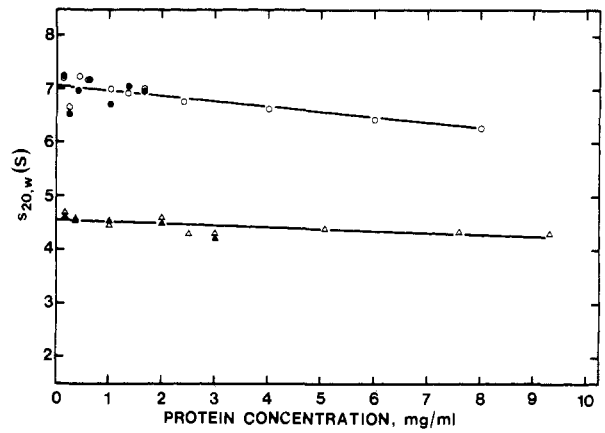

FIGURE 1: Concentration dependence of the sedimentation velocity of succinyl-CoA synthetase. Values of $s_{20,w}$ for *E. coli* succinyl-CoA synthetase were calculated by the methods of midpoint (○) and equivalent boundary position (●). The corresponding data for porcine heart muscle succinyl-CoA synthetase are shown as midpoint method (△) and equivalent boundary position (▲). The buffer used was 50 mM potassium phosphate–0.2 mM EDTA, pH 7.4, and the temperature was 5 °C.

Table I: Sedimentation Coefficients of Succinyl-CoA Synthetases As Determined by Active Enzyme Centrifugation

SCS source	loading concn (μg/mL)	$s_{20,w}$ (S)
E. coli	2	6.4
	5	6.4
	10	6.8
	15	6.6
porcine heart muscle	5	4.5
	10	4.6
	24	4.6

Results and Discussion

Preparatory to active enzyme centrifugation by which sedimentation velocity is measured at assay concentrations of enzyme, we performed conventional sedimentation velocity experiments over a range of concentrations of both *E. coli* and porcine heart succinyl-CoA synthetase. The results, shown in Figure 1, illustrate the concentration dependence of the apparent sedimentation coefficients. Here, sedimentation velocities were calculated separately by both simple midpoint measurements and by equivalent boundary position (Teller, 1973). Since the latter method effectively surveys all of the material throughout the boundary and the values obtained with the two methods overlap well, we conclude that the boundaries for each of the enzymes are symmetrical with no trends that would be indicative of heterogeneity or size distribution. The data, fitted by linear least squares, give intercepts at zero concentration ($s°$) of 7.04 S for *E. coli* SCS and 4.55 S for the porcine heart enzyme. These values may be correlated to those of tetrameric and dimeric dehydrogenase molecules respectively: lactate dehydrogenase from dogfish has a M_r of 146 200 and $s°_{20,w}$ of 7.54 S, while porcine malate dehydrogenase has a M_r of 74 900 and $s°_{20,w}$ of 4.53 S (Squire & Himmel, 1979), suggesting that the succinyl-CoA synthetases have comparable overall shapes. By use of the equation $s = s°(1 - k_sC)$ to characterize the data, the resulting k_s values

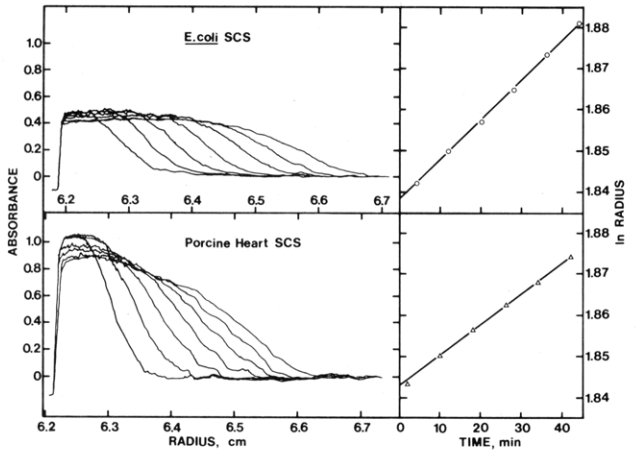

FIGURE 2: Active enzyme centrifugation of succinyl-CoA synthetase from *E. coli* and from porcine heart muscle. The left panels show tracings of superimposed successive scanner records, taken at 8-min intervals. Sedimentation is from left to right. The right panels show plots of the logarithm of the radial position of the maxima of difference curves calculated from successive scanner records vs. the mean time of corresponding scans. With the addition of the components for activity measurement (see Experimental Procedures), buffer and temperature were as for Figure 1.

were 14 and 7 mL/g for the bacterial and heart enzymes, respectively, both values in the typical range for nonreacting globular proteins (Teller, 1973).

Examples of active enzyme centrifugation runs for both enzymes are shown in Figure 2, and the results of several runs at different loading concentrations are summarized in Table I. The data clearly establish that differences in the molecular sizes between the two types of SCS persist to low concentrations that are used for activity measurement.

The technique of active enzyme centrifugation is a powerful tool for the investigation of sedimentation properties of enzymes at low concentrations, but great attention must be given to the avoidance of artifact or error. For example, it was necessary to ensure that the catalytic activity of the band of active enzyme did not significantly deplete the substrates or the DTNB during its passage through a given volume element. To this end, all substrates were present at initial concentrations ranging upward from 16 K_m, and we ensured that the recorded absorbance changes were low compared to those expected for completion of the reaction (see Experimental Procedures). The use of low temperature (5 °C), although necessitating significant corrections of observed sedimentation velocity to standard conditions, was beneficial in maintaining stability of the enzymes and their labile substrate, succinyl-CoA. Further to this point, the linear plots of the logarithm of the peak of the calculated difference curves between successive traces vs. time (see right panels of Figure 2) confirm that the activity of the enzyme is uniform throughout the run and that the active component sediments in a single homogeneous band. Three methods were used for the calculation of sedimentation velocity from these data (midpoint, equivalent boundary position, and the constructed difference curves). These calculations gave no significant differences in resultant values for sedimentation coefficients, again attesting to the symmetry of the boundaries with no trend to high values (aggregation) for the heart enzyme nor to low values (dissociation) for the bacterial species.

In order to relate the observed sedimentation coefficients to molecular weights, it was necessary to complement the sedimentation velocity measurements by appropriate sedimentation equilibrium experiments. Series of sedimentation equilibrium runs were performed with both enzymes, a variety

Table II: Comparison of Molecular Size of Succinyl-CoA Synthetase from *E. coli* and from Porcine Heart Muscle

physicochemical parameter	*E. coli* SCS	porcine heart muscle SCS
apparent M_r by sedimentation equilibrium	139 000 (9.5 mg/mL)	78 000 (9.3 mg/mL)
apparent M_r by gel filtration	136 000[a]	77 000[b]
M_r as calculated from amino acid sequence $(\alpha_2\beta_2)$[c]	142 068	
$s_{20,w}$ (S)	6.3 (8 mg/mL)	4.3 (9.3 mg/mL)
$s^{\circ}_{20,w}$ (S)	7.04	4.55
av $s_{20,w}$ by AEC (S)	6.6	4.6

[a] O'Connor (1982). [b] Bild et al. (1980). [c] Buck et al. (1985).

of loading concentrations and rotor speeds being used. Here, the points represent the apparent weight-average molecular weight at the indicated concentration, as calculated by a sliding least-squares line through each of five successive pairs of data. For both enzymes, particularly *E. coli* SCS, the low concentration ranges show anomalous molecular weight distributions similar to those reported earlier (Krebs & Bridger, 1974) (see below). At concentrations over 2 mg/mL, however, *E. coli* SCS behaves as a tetramer with a molecular weight near 140 000, and the porcine enzyme is a dimer of about M_r 78 000, showing no evidence of any tendency to form tetramers even at concentrations over 10 mg/mL.

Table II shows a summary of the molecular weight data with correlated sedimentation coefficients. The results obtained from our sedimentation equilibrium experiments agree very well with the apparent molecular weights obtained by gel filtration. It is important to note that the latter were obtained with columns that had been preequilibrated with substrates, so that the apparent sizes represent those of the enzyme undergoing catalysis, although at much higher concentrations than we have used for active enzyme centrifugation. Taken together, the data provide convincing evidence that *E. coli* SCS acts as a tetramer at assay concentrations and that the heart enzyme acts only as a dimer. First, for both enzymes, the values of $s_{20,w}$ obtained from active enzyme centrifugation agree well with those of $s^{\circ}_{20,w}$ that were obtained by conventional sedimentation velocity runs. Second, values for $s_{20,w}$ at high concentration (8–10 mg/mL) may be correlated to the molecular weights obtained by sedimentation equilibrium at similar concentrations. Thus, it is established that molecular weights of the active enzymes are the same as those measured for concentrated solutions.

This conclusion aids in our interpretation of the anomalous size distribution observed at low concentrations in the sedimentation equilibrium experiments. The distribution clearly does not reflect an equilibration of dissociating forms, since such a system must give rise to equivalent or overlapping distributions regardless of rotor speed or loading concentration (Teller, 1973). Significantly, the active enzyme centrifugation data show no indication of any trend to dissociation, as would be reflected by a change in the s values during sedimentation, implying that low molecular weight material that may contribute to the anomalous size distribution seen in Figure 3 does not contribute to the population of active enzyme molecules. The anomalous distribution, noted in earlier sedimentation equilibrium studies of *E. coli* succinyl-CoA synthetase (Krebs & Bridger, 1974; O'Connor, 1982), is thus of small significance to the conclusions reached in this study and remains unexplained. Clearly, possible factors that may contribute include preexisting size heterogenetiy in our samples or development

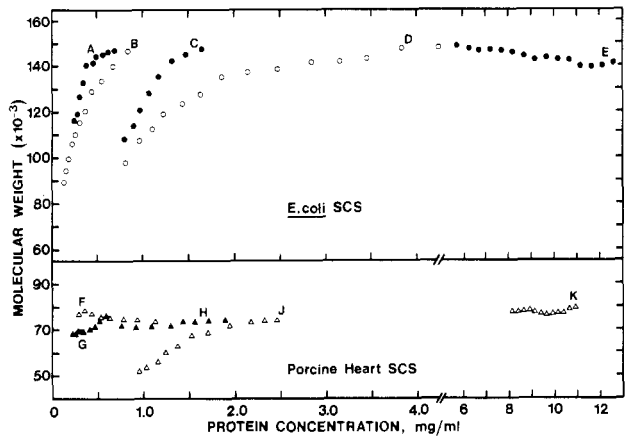

FIGURE 3: Concentration dependence of the apparent weight average molecular weight for succinyl-CoA synthetases from *E. coli* and from porcine heart muscle as determined by sedimentation equilibrium. The open and closed point styles differentiate separate equilibrium experiments. For clarity, only every second point of the resultant molecular weight distribution has been plotted. The various speeds and loading concentrations of the two enzyme species were as follows: for *E. coli* succinyl-CoA synthetase (data curves A–E), (A) 6400 rpm, 0.5 mg/mL, (B) 11 000 rpm, 0.5 mg/mL, (C) 6400 rpm, 1.4 mg/mL, (D) 10 000 rpm, 2.5 mg/mL, and (E) 6000 rpm, 9.5 mg/mL; for porcine heart succinyl-CoA synthetase (data curves F–K), (F) 11 000 rpm, 0.8 mg/mL, (G) 9000 rpm, 0.3 mg/mL, (H) 11 000 rpm, 1.5k mg/mL, (J) 9000 rpm, 1.5 mg/mL, and (K) 4400 rpm, 9.3 mg/mL. The buffer was 50 mM potassium phosphate–0.2 mM EDTA, pH 7.4, and the temperature was 5 °C.

of heterogenetiy from irrevesible dissociation and inactivation during the prolonged equilibrium runs.[4]

The conclusions that we have reached have a bearing on possible rationalizations for the quaternary structures of the two species of succinyl-CoA synthetase. Many lines of evidence, including half-site behavior with respect to enzyme phosphorylation (Moffet et al., 1972; Bridger, 1974; Wolodko et al., 1983), ^{31}P NMR studies (Vogel & Bridger, 1982, 1983), hybrid enzyme formation (Wolodko et al., 1981; O'Connor-McCourt & Bridger, 1985), and ^{18}O exchange kinetics (Bild et al., 1980), point to cooperative interactions between the two $\alpha\beta$ halves of the tetrameric *E. coli* enzyme. Here, we and others have argued that unfavorable catalytic events at one active site may be boosted by favorable events (e.g., phosphorylation) at the neighboring active site. Thus, alternating action of the two halves of an asymmetric enzyme molecule with cooperative conformational changes linked to catalysis could make the tetrameric enzyme a more effective catalyst than an equivalent dimer. In this context, it is then appropriate to consider the properties of porcine heart succinyl-CoA synthetase, which we have shown here to be a simple $\alpha\beta$ dimer at all concentrations. This enzyme has a catalytic activity that is comparable to that of the *E. coli* enzyme (its specific activity is about 30 units/mg, compared to a value of 45 units/mg for the *E. coli* species). Thus, it obviously functions well without the opportunity for favorable interactions between like sites. One line of evidence that we have considered to support alternating sites cooperativity for the *E. coli* enzyme is the stimulatory effect of ATP on the rate of dethiophosphorylation of the enzyme in the presence of succinate and CoA, which we suggested was the result of phosphorylation of the neighboring active site (Wolodko et al., 1983). Nishimura and Mitchell (1985), however, have shown an equivalent stimulatory effect of GTP on the dethiophosphorylation of the heart enzyme and have suggested that this results from interaction at a second nucleotide binding site. Our present results are consistent with that interpretation for the pig heart enzyme. It may be significant that the porcine heart enzyme is significantly larger (M_r 78 000) than one $\alpha\beta$ half of the *E. coli* species (M_r 71 000). This difference could represent the presence of an extra domain in the heart enzyme that contains a binding site for GTP, obviating the benefits that may be available to a tetramer. Alternatively, the size difference of the subunits may reflect the fact that the mature heart subunits could contain signal sequences to direct their mitochondrial import, structures that would have no counterpart in the procaryotic species of enzyme.

[4] The enzyme is stable for equivalent time periods at the temperature of the run, showing no changes in activity nor homogeneity on polyacrylamide gel electrophoresis. Instability in the ultracentrifuge, in the absence of substrates and at elevated pressures, may be a factor, however.

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Registry No. Succinyl-CoA synthetase (GDP forming), 9014-36-2; succinyl-CoA synthetase (ADP forming), 9080-33-5.

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New Hydrophilicity Scale Derived from High-Performance Liquid Chromatography Peptide Retention Data: Correlation of Predicted Surface Residues with Antigenicity and X-ray-Derived Accessible Sites†

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ABSTRACT: A new set of hydrophilicity high-performance liquid chromatography (HPLC) parameters is presented. These parameters were derived from the retention times of 20 model synthetic peptides, Ac-Gly-X-X-$(Leu)_3$-$(Lys)_2$-amide, where X was substituted with the 20 amino acids found in proteins. Since hydrophilicity parameters have been used extensively in algorithms to predict which amino acid residues are antigenic, we have compared the profiles generated by our new set of hydrophilic HPLC parameters on the same scale as nine other sets of parameters. Generally, it was found that the HPLC parameters obtained in this study correlated best with antigenicity. In addition, it was shown that a combination of the three best parameters for predicting antigenicity further improved the predictions. These predicted surface sites or, in other words, the hydrophilic, accessible, or mobile regions were then correlated to the known antigenic sites from immunological studies and accessible sites determined by X-ray crystallographic data for several proteins.

It is now thought that the entire surface of a protein can be considered antigenic when peptide fragments of the protein surface are used as immunogens or a different species is used as the immunizing host (Green et al., 1982; Benjamin et al., 1984). Antigenic sites are defined as those residues of a native protein that are bound by antibodies raised to a native protein, native protein fragments, or synthetic peptides. By definition, since antigenic sites are those recognized by antibodies, it is most likely that these sites are accessible or on the surface of a protein, and these regions are probably more mobile than interior regions. Since these sites are on the surface, they are probably hydrophilic. Indeed, algorithms for hydrophilicity and accessibility have been used to predict antigenicity.

We have experimentally determined a new hydrophilicity scale derived from the contribution in high-performance liquid chromatography (HPLC)[1] of each amino acid side chain to the retention time of model synthetic peptides, Ac-Gly-X-X-$(Leu)_3$-$(Lys)_2$-amide, where X was substituted by the 20 amino acids found in proteins. This new set of hydrophilicity parameters was used in a modified Hopp and Woods (1978) algorithm to predict which areas of a protein are on the surface. It was found that these predicted surface sites correlate well with the known antigenic sites for myoglobin, lysozyme, cytochrome *c*, and influenza hemagglutinin. An excellent correlation was also shown for accessible sites determined by X-ray data for myoglobin, lysozyme, cytochrome *c*, bovine trypsin, rat mast cell protease, and *Streptomyces griseus* trypsin.

Experimental Procedures

Materials. Unless otherwise stated, chemicals and solvents were reagent grade. Diisopropylethylamine (DIEA), dichloromethane (CH_2Cl_2), and trifluoroacetic acid (TFA) were redistilled prior to use. Picric acid was dissolved in CH_2Cl_2 and dried over magnesium sulfate. Acetonitrile (HPLC grade) was obtained from Fisher Scientific, Fairlawn, NJ. Double-distilled water was purified by passage through a Milli-Q water purification system (Millipore Corp., Bedford, MA). Poly(styrene-*co*-divinylbenzene) benzydrylamine hydrochloride resin (0.75 mmol of NH_2/g of resin) and poly(styrene-*co*-divinylbenzene) chloromethyl resin (~1.0 mmol of Cl/g of resin) were purchased from Beckman Instruments, Inc., Palo Alto, CA, and Pierce Chemical Co., Rockford, IL, respectively. *tert*-Butyloxycarbonyl (Boc) amino acids were purchased from Vega Biochemicals (Tucson, AZ), Bachem Fine Chemicals, Inc. (Torrance, CA), Beckman Instruments, Inc. (Palo Alto, CA), and the Protein Research Foundation (Japan).

Apparatus. Peptide synthesis was carried out on a Beckman peptide synthesizer, Model 990. The HPLC instrumentation was composed of a Spectra-Physics SP8700 solvent delivery system and SP8750 organizer module, combined with a

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[1] Abbreviations: HPLC, high-performance liquid chromatography; RPC, reversed-phase high-performance liquid chromatography; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid; BOC, *tert*-butyloxycarbonyl.

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