Conformational Changes and Aggregation in Phosphoribosyladenosine Triphosphate Synthetase. Ligand Effects on Hydrogen Exchange and Hydrophobic Probe Uptake*

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ABSTRACT: Phosphoribosyladenosine triphosphate synthetase from *Escherichia coli* is inhibited by AMP, phosphoribosyladenosine triphosphate, and histidine, and particularly by combinations of one of the nucleotides and histidine. We have monitored the enzyme for conformational changes when the specific ligands are bound. High concentrations of histidine alone did not influence the exchange of tritium-labeled hydrogen from the enzyme, AMP inhibited the exchange, while combinations of AMP and histidine were more efficient inhibitors of the exchange. Temperature increase increases the tendency of the enzyme to be inhibited at high enzyme concentrations, presumably by aggregation. For this reason we studied the effect of temperature on the interaction of the enzyme with the hydrophobic probe 8-anilinonaphthalene-1-sulfonic acid (ANS). ANS uptake by the synthetase consists of two phases, one rapid and one slow. Over a broad range of ANS concentrations more ANS was bound to the synthetase at a lower than at a higher temperature. At millimolar concentrations of ANS, the binding of a large number of ANS molecules could be demonstrated at 2° by binding studies. At 21° far less ANS was bound to the enzyme. AMP inhibited the binding of ANS at 10°, but only to a small extent at 30°. Histidine inhibited ANS uptake at both temperatures. It is concluded that histidine mainly acts by stabilizing one specific aggregate form of the enzyme, while AMP may affect the conformation of the enzyme more extensively.

Phosphoribosyladenosine triphosphate synthetase[1] from *Escherichia coli* is regulated by several interesting mechanisms: synergistic control by energy charge and feedback inhibition by histidine; inhibition by AMP, inhibition by PRibATP (the immediate product in the reaction catalyzed by the synthetase), and synergistic inhibition by histidine and PRibATP (Klungsöyr *et al.*, 1968; Klungsöyr and Atkinson, 1970). We have recently suggested a model for the enzyme, based on the available information about its kinetic behavior, and its sedimentation properties (Klungsöyr and Kryvi, 1971; Kryvi and Klungsöyr, 1971). Our model is perhaps best classified as sequential (Koshland *et al.*, 1966) rather than as a symmetrical two-state model (Monod *et al.*, 1965). In this connection it is important to be able to distinguish between the various forms of the enzyme that have sedimentation coefficients near 8.9 S, but which for kinetic reasons seem to be different depending upon which ligand is present.

The conformational probes used in this work to study ligand effects were chosen to reflect different properties. Tritium–hydrogen exchange is a good relative measure of the degree of "looseness" of the enzyme molecule as influenced by its ligands, and the binding of ANS gives information about the accessibility of hydrophobic sites or zones on the enzyme (Stryer, 1968; Radda, 1971).

An interesting property of this enzyme is its inhibition at high enzyme concentrations. The inhibition is most pronounced at higher temperatures. Our interpretation of this finding was that the enzyme is inhibited by aggregation, and that hydrophobic groups take part in the aggregation (Kryvi and Klungsöyr, 1971). For that reason ANS seemed promising for studying the effects of temperature on the enzyme conformation.

Materials and Methods

PRibATP synthetase was prepared as described previously (Klungsöyr and Atkinson, 1970) including the final dialysis step.

Fluorescence measurements were carried out on a Farrand spectrofluorimeter, Mark I. The temperature was controlled by circulating water from constant-temperature baths through the cuvet holder. The light path was 10 mm.

8-Anilinonaphthalene-1-sulfonic acid (Eastman) was recrystallized as the magnesium salt (Weber and Young, 1964). When the purified product was passed through Sephadex columns, a darkly colored zone remained on the upper part of the gel. For this reason all ANS solutions used for gel binding studies were first passed through a short column of Sephadex G-25, medium.

Basal buffer (Klungsöyr and Kryvi, 1971) consisted of 10 mM imidazole-HCl buffer (pH 7.2) and 0.5 ml of 2-mercaptoethanol/l.

The binding of ANS to PRibATP synthetase was studied by the method of Hummel and Dreyer (1962). Double-strength ANS solutions were prepared either in 0.1 M Tris-HCl buffer (pH 8.5), containing 0.1 M NaCl and 0.5 ml of 2-mercaptoethanol/l., or in basal buffer with 0.1 M NaCl. The enzyme was dialyzed against basal buffer + 0.1 M NaCl, equal volumes of double-strength ANS solutions were added, and the mixtures were preincubated for 60 min at ice–water temperature, or for 45 min at room temperature (21°). Sephadex G-25, medium, was packed in columns measuring 190 × 8 mm, and

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[1] Abbreviations used are: PRibPP, 5-phospho-D-ribosyl α-pyrophosphate; PRibATP, *N*-1-(5-phosphoribosyl)adenosine 5′-triphosphate; ANS, 8-anilinonaphthalene-1-sulfonic acid.

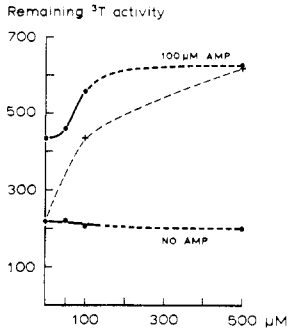

FIGURE 1: Effect of histidine and AMP on the tritium–hydrogen exchange in PRibATP synthetase. The exchange-in was carried out for 45 hr at 2°, in basal buffer containing 0.1 M NaCl and at an enzyme concentration of 10 mg/ml. The ^3H activity was 10 mCi/ml. The exchange-out was carried out for 24 hr at 0°. The enzyme concentration was 0.8 mg/ml, and the solvent was basal buffer containing 0.1 M NaCl with the appropriate ligands added: (●) concentrations of histidine (0 or 100 μM AMP); (+) concentrations of AMP (no histidine). Each point represents the average of two determinations.

TABLE I: Effect of AMP and Histidine on Back-Exchange from ^3H-Labeled PRibATP Synthetase.[a]

Ligands (μM)		^3H Act. (cpm)		
Histidine	AMP	Conditions at Back-Exchange		
		4 hr 20°	24 hr 0°	48 hr 0°
0	0	414	888	530
500	0	463	769	489
0	100	541	1009	618
500	100	1226	1349	1136

[a] The exchange-in took place at a protein concentration of 8 mg/ml in basal buffer containing 0.1 M NaCl. The ^3H activity was 5 mCi/ml. The exchange-out was carried out with a protein concentration of 2.5 mg/ml, at ice–water or room temperature, in the presence of the ligands specified in the table. The values given are the averages of two separate determinations.

equilibrated with the respective ANS solutions, which were prepared by diluting the double-strength solutions with basal buffer containing 0.1 M NaCl. The protein–ANS mixtures were added to the columns and washed through with identical concentrations of ANS. Fractions of 5 drops were collected. The fractions were diluted with distilled water and the absorbance at 350 nm was determined. Protein was determined in the tubes exhibiting fluorescence, and the number of moles of ANS bound per mole of enzyme (200,000 g) was calculated, based on a millimolar absorbance of 5.0 at 350 nm. No systematic differences in the results were observed whether the reaction mixture contained Tris or not.

Hydrogen-exchange studies were carried out on the microfiltration device described by Paulus (1969) using Diaflo PM 10 filters (Amicon). Concentrated enzyme solutions were dialyzed against basal buffer + 0.1 M NaCl and then incubated in an ice bath for approximately 48 hr with tritiated water added. The protein solution was then passed through a column of Sephadex G-25, medium, the protein rapidly localized by counting samples of the eluate, and the highest fractions combined. For out-exchange the combined fractions were rapidly diluted with basal buffer + 0.1 M NaCl containing the appropriate ligands. From the time when the protein was separated from its exchange-in environment on the gel column, until the dilution process was completed, approximately 40 min passed, during which time the labeled protein was kept at cold room or ice temperature. After completion of the exchange-out incubation, 20 μl of enzyme solution containing 15–50 μg of protein was added to the channels of the filtration device, and 100 μl of the corresponding buffer ligand solution was layered on top. Filtration was carried out under a pressure of 1.5 kg/cm² of nitrogen, at the temperature required. When all filters were dry, the buffer corresponding to each filter was injected through the rinse channel, and finally the underside of each filter was blown dry with a stream of air. Remaining ^3H activity was counted in a liquid scintillation counter.

Protein was determined by the method of Klungsöyr (1969).

Determination of PRibATP synthetase activity was carried out on a Shimadzu MPS-50L spectrophotometer by measuring the change in absorbance at 290 nm at 30°. The complexes of synthetase and ANS were often sensitive to dilution, and these preparations were then assayed as they were obtained from the column. For reference, an activity *vs.* enzyme concentration curve was prepared with the native enzyme, and the activities of the ANS-containing preparations were then compared to that of the native enzyme at the corresponding protein concentration.

PRibATP was prepared according to Ames *et al.* (1961) with the modification described by Klungsöyr and Kryvi (1971).

Tritiated water was obtained from the Radiochemical Centre, Amersham, Great Britain. AMP, PRibPP, ATP, and purified pyrophosphatase were the products of Sigma.

Results

Tritium Exchange. The back-exchange of tritium from labeled PRibATP synthetase was studied with the ultrafiltration technique described by Paulus (1969), with minor adaptations as described in the Materials and Methods section. In a typical experiment in which the back-exchange was carried out for 24 hr at ice-bath temperature with 1.7 mg of enzyme/ml, four separate determinations of the remaining tritium activity were made for each experimental condition. The back-exchange in all cases took place in basal buffer + 0.1 M NaCl, with AMP or histidine added as indicated. The average values and the standard deviations were expressed as gram-atoms of tritium-labeled hydrogen remaining per 200,000 g of enzyme: no addition, 61.0 ± 1.9 g-atoms; 200 μM histidine, 61.5 ± 2.6 g-atoms; 100 μM AMP, 86.5 ± 6.5 g-atoms, 200 μM histidine + 100 μM AMP, 119.5 ± 15.3 g-atoms.

The data in Table I summarize the results of experiments in which the back-exchange took place for different periods of time, and at two different temperatures. The extent of isotope loss after 4 hr at room temperature corresponds roughly to that after 48 hr at 0°. An exchange time of 24 hr at ice-bath temperature gave higher "blank" values, but in all cases the effects of the ligands were similar. Saturating concentrations of histidine (500 μM) had no measurable effect on the loss of tritium activity from the enzyme, while 100 μM AMP had some effect, and a combination of the two ligands inhibited the back-exchange considerably.

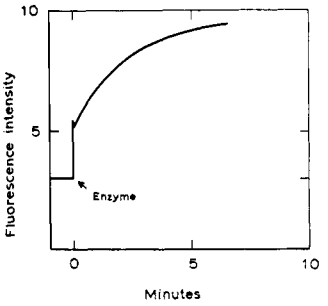

FIGURE 2: Copy of recorder tracing showing increase in fluorescence intensity after addition of PRibATP synthetase to a solution of ANS. The reaction mixture contained: 0.1 M Tris-HCl buffer (pH 8.5), 0.1 M KCl, 1 mM $MgCl_2$, and 50 μM ANS. The exciting wavelength was 390 nm, and the light emission was measured at 490 nm, at 30°. At the point indicated, enzyme was added to a concentration of 50 μg/ml in a total volume of 2.5 ml.

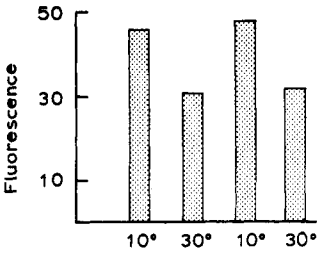

FIGURE 3: Effect of temperature on the fluorescence increase upon binding of ANS to PRibATP synthetase. The reaction mixture contained: 0.1 M Tris-HCl buffer (pH 8.5), 0.1 M KCl, 1 mM $MgCl_2$, 25 μM ANS, and 20 μg of synthetase protein/ml. The mixture was incubated at 10° for 1 hr, a sample was removed for reading in the spectrofluorimeter at 10°, and the rest of the mixture was transferred to a 30° water bath where it was gently stirred until temperature equilibrium was obtained. A sample was read at 30° and the mixture was again cooled to 10°, read, and reheated to 30° for the final reading. The intensities shown are net values, with the fluorescence of ANS subtracted.

Similar results are shown in Figure 1, where three different concentrations of histidine were tested in the presence and absence of 100 μM of AMP. The effect on back-exchange of a higher concentration of AMP (500 μM) was also tested. It may be concluded that under the conditions of these experiments, histidine had no measurable effect on the tritium–hydrogen exchange in PRibATP synthetase, while AMP inhibited the exchange. When the two ligands were acting in combination, histidine increased the inhibition caused by AMP.

Interaction between the Synthetase and 8-Anilinonaphthalene-1-sulfonic Acid. PRibATP synthetase forms complexes with ANS. The interaction may be demonstrated by measuring the increase in fluorescence of the hydrophobic probe, or by direct demonstration of ANS binding in gel filtration experiments. The recorder tracing from an experiment in the spectrofluorimeter which is copied in Figure 2 demonstrates that the ANS synthetase interaction consists of two phases with respect to time, a fast phase is followed by a slow process. This is reminiscent of the interaction of ANS with membrane preparations (Radda, 1971). During the increase in fluorescence the exitation maximum is shifted from 370 to 390 nm, and the emission maximum is shifted from 515 to 490 nm.

The initial rates of the slow phase of fluorescence increase are difficult to measure accurately. Rather the reciprocal of the time required to produce an arbitrary change in fluorescence may be used. After incubation for 30–60 min an equilibrium intensity is approached, after which the fluorescence changes little with time. In the experiments reported here the effects of specific ligands and temperature on the equilibrium fluorescence were studied. However, it is interesting to note that temperature and ligands influence the equilibrium fluorescence and the "rate" of fluorescence increase in different ways.

As shown in Figure 3 the equilibrium fluorescence is temperature dependent, and considerably higher at 10° than at 30°. The reaction mixture may be cooled and heated several times and show increase and decrease in fluorescence. The fluorescence of ANS in buffer changes very little under the same conditions. AMP abolishes, or at high concentrations reverses the temperature dependence of the synthetase–ANS fluorescence. Changes in quantum efficiency with temperature, therefore, can probably not explain the fluorescence changes. Rather, the dissociation constant of the synthetase–ANS complex may increase with increasing temperature, or the increase in temperature may cause a reduction in the number of ANS binding sites.

The latter possibility is of considerable interest, since it has been shown in this laboratory that the synthetase is inhibited at high enzyme concentrations, and that the inhibition increases with increasing temperature (Kryvi and Klungsöyr, 1971). The inhibition is possibly caused by aggregation in which hydrophobic groups participate.

The fluorescence was measured as a function of ANS con-

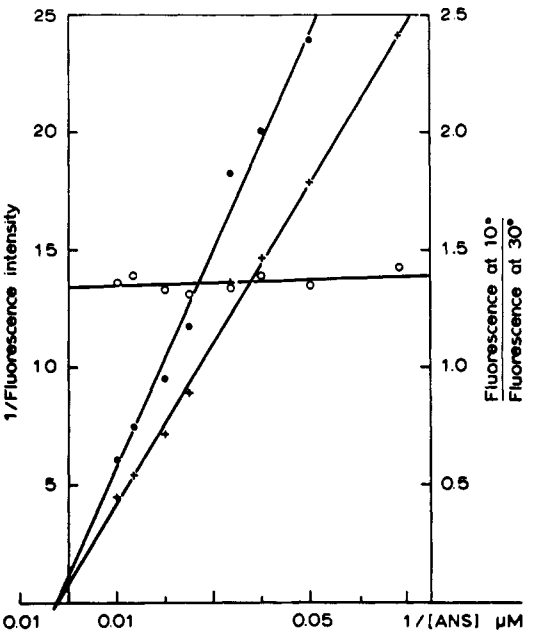

FIGURE 4: Reciprocal net fluorescence intensity plotted against the reciprocal of the ANS concentration, at 10° (+) and at 30° (●), and ratios between the net fluorescence intensities at 10 and 30° plotted against the reciprocal of the ANS concentraton (○). The reaction mixtures contained: 0.1 M Tris-HCl buffer (pH 8.5), 0.1 M KCl, 10 mM $MgCl_2$, 50 μg/ml of PRibATP synthetase, and ANS as shown. Preincubation times from 60 to 90 min at the appropriate temperatures. The fluorescence intensities were corrected for ANS fluorescence and for absorption of exciting light through the cuvet. The absorption correction factor at each concentration was obtained from

$$\int_0^x I dx = I_0(1 - e^{-kcx})/kc$$

where I is exciting light intensity at the light path x, c is ANS concentration, and k the experimentally determined absorbance coefficient of ANS at the exciting wavelength, 390 nm.

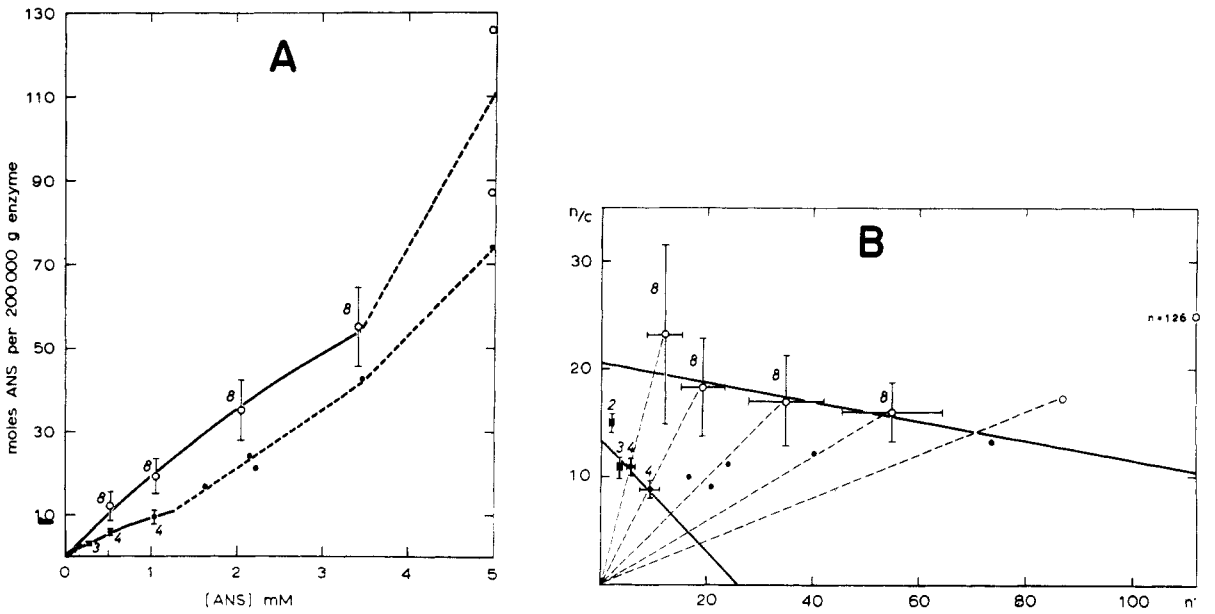

FIGURE 5: Effects of ANS concentration and temperature on the binding of ANS to PRibATP synthetase. Experiments were carried out as described in the Materials and Method section at 2° (○), and at 21° (●). Average values, number of experiments, and standard deviations are shown in the figure. The maximal protein concentration in the eluates from the columns varied between 1 and 4 mg per ml. In Figure 5B the data were replotted as a Scatchard plot. Abscissa: the number of moles of ANS bound per 200,000 g of enzyme = n. Ordinate: n divided by the concentration of ANS. The broken lines in the Scatchard plot represent equal concentrations of ANS.

centration with constant enzyme concentration at the two temperatures, 10 and 30° (Figure 4). The ratio between the fluorescence intensities at the two temperatures remained almost constant over a considerable concentration range, and extrapolation of the inverse fluorescence intensity to saturation values gave different intercepts at the two temperatures. Because of the high absorbance of the probe at the exciting wavelength we could not measure the fluorescence intensity at ANS concentrations close to the value of the dissociation constant of the complex. A rough estimate of the dissociation constant under these conditions is 0.3–0.5 mM at both temperatures. The saturation fluorescence intensity was compared to a standard of bovine serum albumin, and the number of sites available for ANS binding was estimated to 9 and 12 per 200,000 g of enzyme at 30 and 10°, respectively.

At higher probe concentrations it is possible to show a direct binding of ANS to the synthetase by the gel filtration method. With relatively large amounts of enzyme a sufficiently big difference in absorbance at 350 nm is obtained between fractions containing protein and ANS and the fractions containing only ANS. Considerable errors are involved in these determinations, but judging from differences between duplicate determinations within each experiment, the variation in the results is for the most part not of analytical origin. The results are presented in Figure 5. It is clear from the data in Figure 5 that more ANS is bound to the enzyme at 2° than at 21°, and also that this high binding at the lowest temperature is not caused by a decreased dissociation constant, but rather by more available sites for ANS binding. The plot (Scatchard, 1949) in Figure 5B has its point arranged in an upward concave pattern, indicating the existence of classes of ANS binding sites with different dissociation constants. This is evident also from other data. In Figure 2 the fast and the slow phase of the increase in fluorescence probably represent different kinds of ANS binding sites. From the data in Figure 4, dissociation constants of the order of 0.5 mM were estimated, while the lines drawn in Figure 5B correspond to 2 mM at 21° and 12 mM at 2°. At 21° 25 sites for ANS binding were available per 200,000 g of enzyme, while almost 10 times as many were exposed at 2°, judging from the arbitrarily drawn lines in Figure 5B. When the concentrations of ANS increase above certain levels, about 3.5 mM at 2° and about 1.1 mM at 21°, unspecific complex formation seems to take place, as judged by the positive slopes in the Scatchard plots.

This unspecific ANS binding at high probe concentrations might be caused by extensive denaturation of the enzyme, but this is apparently not the case as shown in Figure 6. In this experiment the enzyme was treated with different ANS concentrations at 2°, passed through gel columns with the same ANS concentrations, whereupon the enzymatic activity and the number of bound ANS molecules per mole of enzyme were determined. The activity decreased gradually as the number of bound probe molecules increased. At the highest ANS concentration tested, 5 mM, the activity was very low, and more ANS was bound than expected from the pattern established with the lower concentrations. However, the loss of activity at 5 mM ANS concentration was reversible. When a sample identical with that used in the binding experiment was passed through a gel column containing no ANS, the enzyme was eluted with no bound probe, and the activity was not much below that obtained in a similar control experiment without ANS.

When the synthetase was incubated in the presence of 5 mM ANS and low concentrations of the detergent polyoxyethylene lauryl ether (Brij 35) and then passed through a gel column containing no ANS, some of the probe remained bound to the enzyme. The complex had a broad absorbance peak between 390 and 350 nm, and its ANS content was roughly estimated to 6 moles/200,000 g of enzyme. This complex has nearly the same activity as the native enzyme when assayed at 30° and high enzyme concentration, but the complex showed no increase in activity upon dilution.

The specific ligands of the synthetase control the extent of

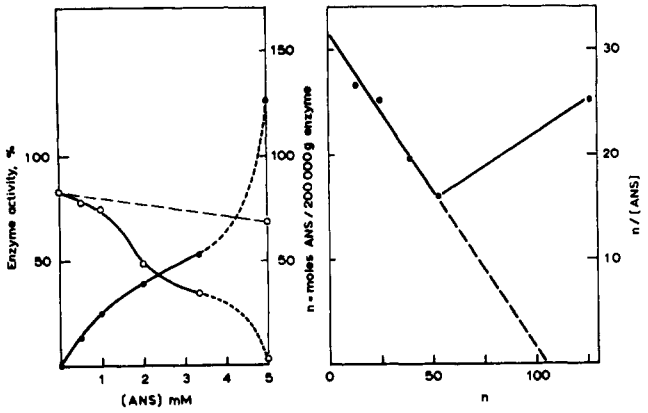

FIGURE 6: Synthetase activity of ANS enzyme complexes prepared in basal buffer + 0.1 M NaCl at 2°. Left graph: (●—● - - - ●) the number of moles of ANS bound per mole (200,000 g) of enzyme as a function of the ANS concentration. (○—○ - - - ○) Synthetase activity at 30° of the ANS containing preparations compared to the native enzyme. (○ - - -○) Activities of ANS-free preaprations. The left point represents a control experiment with no ANS added, and the right point a preparation which was preincubated with 5 mM ANS in ice for 90 min and then passed through a column without ANS. Right graph: a Scatchard plot of the binding data. The data in Figure 6 are included in Figure 5.

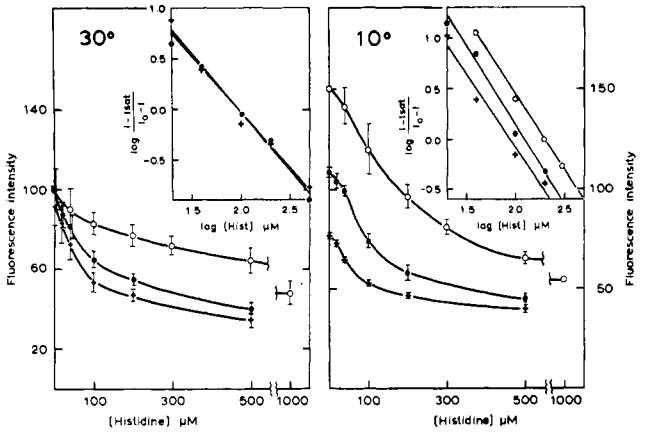

FIGURE 7: The effect of histidine and AMP on the net fluorescence intensity of the ANS–PRibATP synthetase complex at 10 and 30°. The reaction mixtures contained: 0.1 M Tris-HCl buffer (pH 8.5), 0.1 M KCl, 1 mM $MgCl_2$, 25 μM ANS, and histidine as shown. The enzyme was finally added to a concentration of 25 μg/ml in a total volume of 2.5 ml. After addition of the enzyme the reaction mixtures were incubated at the appropriate temperature for 60 min before the readings were started. (○) No AMP; (●) 100 μM AMP; (+) 500 μM AMP. Average values and standard deviations are shown, at 10° for four experiments (two different enzyme preparations), at 30° for three experiments (two different enzyme preparations).

binding of ANS. This is illustrated in Figure 7 for AMP and histidine at two temperatures. At 30° AMP alone has little effect on the fluorescence intensity of the complex, but increases the effect of histidine. A Hill plot for the effect of histidine alone could not be constructed because of difficulties in extrapolating to the fluorescence with saturating histidine concentrations, but in the presence of AMP the effect of histidine on complex fluorescence seems to be of hyperbolic nature, as shown by the Hill plot slopes (inset) which are quite near 1. At 10° AMP by itself inhibited ANS binding considerably, but the synergistic effect of AMP on histidine inhibition was less evident than at 30°. At 10° cooperativity in the histidine effect on ANS synthetase complex formation is indicated by Hill plot slopes of 1.6. The same slopes were obtained both in the presence and in the absence of AMP. This is in contrast to the observations in kinetic experiments (Krvyi and Klungsöyr, 1971), where high AMP concentrations changed the inhibitory effect of histidine from a cooperative to a hyperbolic pattern.

The product of the synthetase reaction, PRibATP, strongly reduces the rate of binding of ANS to the enzyme as observed by the increase in fluorescence intensity with time. PRibATP is also a substrate for the reverse reaction, and upon addition of the other substrate, PP_i, PRibATP is removed from the reaction mixture. The rate of fluorescence increase then rises to levels similar to those seen in control experiments without PRibATP. This experiment illustrates that actively functioning enzyme molecules are involved in probe binding.

Discussion

The regulatory mechanism of PRibATP synthetase from *E. coli* appears to involve aggregation processes. In the ultracentrifuge, when no ligands are added, several rapidly sedimenting species are seen, with $s_{20,w}$ values of 12.6 S and upward, in addition to some low molecular weight material. In the presence of the inhibitors histidine, AMP, or the product PRibATP, a form with $s_{20,w}$ = 8.9 S appears. High concentrations of ATP also give the 8.9S species (Klungsöyr and Kryvi, 1971). Under conditions when the enzyme sediments predominantly in the 8.9S peak, relatively less of the low molecular weight material seems to be present. Kinetic experiments have shown that the synthetase is inhibited at high enzyme concentrations and that this inhibition is temperature dependent. At 35° the concentration inhibition is very strong, at 25° intermediate, and at 15° relatively weak (Kryvi and Klungsöyr, 1971). Our hypothesis is that the low molecular weight forms of the enzyme are the active species, and that the enzyme is inhibited when specific (8.9 S) or nonspecific aggregates predominate. Hydrophobic forces may be important in these aggregates, and stabilization of the aggregates with increasing temperature would be expected.

In experiments reported here, pairs of temperatures about 20° apart were chosen in order to search for evidence of conformational changes and aggregation at the higher temperature compared to the lower temperature. It was found, by two different techniques, that the amount of hydrophobic probe, ANS, that binds to the synthetase is larger at 2° than at 21°, and larger at 10° than at 30°. It is tempting to speculate that this reflects the tendency of the synthetase to aggregate, and that the hydrophobic areas that bind many ANS molecules are actually involved in the aggregation.

With the fluorescence technique at low concentrations of ANS and enzyme, the specific ligands AMP and histidine inhibited ANS binding. The concentrations of the ligands that were needed to inhibit ANS binding were similar to those needed to inhibit the activity of the enzyme (Klungsöyr and Atkinson, 1970; Kryvi and Klungsöyr, 1971), and to stabilize the 8.9S species in the ultracentrifuge (Klungsöyr and Kryvi, 1971). However, some apparent differences with respect to the cooperativity of the histidine effect require clarification.

The effects of the ligands AMP and histidine on ANS binding may be seen as additional evidence for the hypothesis that the 8.9S species created by the ligands are stable, inhibited forms of the enzyme. Compared to the unspecific higher

aggregates, the 8.9S species have less tendency to dissociate into enzymatically active, ANS binding low molecular weight forms. According to the results shown in Figure 7, the effects of AMP and of increased temperature on ANS binding seem to be non additive. This provides an interesting link between temperature and ligand effects.

The hydrogen-exchange experiments with PRibATP synthetase were designed to test the prediction by our model (Klungsöyr and Kryvi, 1971) that the different ligands of the synthetase stabilize forms of the enzyme that are different, even if they sediment with a similar speed in the ultracentrifuge. The results seem to lead to the conclusion that the 8.9S species stabilized by histidine is different from that stabilized by AMP. At concentrations of histidine high enough to give a high degree of inhibition of enzyme activity, no effect could be detected on the hydrogen exchange. AMP alone had some inhibitory effect on the hydrogen exchange, while a combination of the two ligands inhibited the exchange considerably. In other words, histidine alone seems to have no effect on the degree of "looseness" of the enzyme, while AMP binds to give a tighter form. The temperature did not seem to influence this pattern.

Blasi *et al.* (1971) have recently published a study on the histidine effect on the PRibATP synthetase from *Salmonella typhimurium*. They present evidence that indicates the burying of 12 tyrosyl residues as a result of histidine binding. At the same time, they found no great changes in the secondary structure of the enzyme with circular dichroism techniques. Kryvi, in our laboratory has been unable to show any change in the circular dichroism spectrum by addition of histidine to the *E. coli* enzyme (H. Kryvi, unpublished experiments).

There seems to be a good agreement between the circular dichroism experiments, and our hydrogen-exchange data. Furthermore, the burying of residues upon histidine binding seen in the *Salmonella* enzyme would be in good agreement with our suggested mechanism of aggregation for the *Escherichia* enzyme.

Acknowledgments

During the initial phases of this work (1968–1969) the author visited in Professor D. E. Atkinson's laboratory at the University of California, Los Angeles. I am indepted to Dr. Atkinson for much aid and advice during that period. The technical assistance of Mrs. Trine Herland Tydal is gratefully acknowledged.

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