Immunoglobulin G Disassembly during Thermal Denaturation in Sodium Dodecyl Sulfate Solutions†

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ABSTRACT: Immunoglobulin G solutions in dodecyl sulfate when heated to 100 °C rapidly undergo a thiol–disulfide interchange process which eventually yields the free constituent heavy and light chains as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Evidence for a disulfide interchange mechanism was obtained from studies involving alkylamidation with iodoacetamide and experiments with the disulfide interchange-promoting couple oxidized–reduced glutathione. IgG disassembly was inhibited by the presence of iodoacetamide during heat treatment. Incubations of IgG in nondetergent solutions at 23 °C with 10^{-4} M oxidized–10^{-3} M reduced glutathione did not significantly dissociate it. Inclusion of 6 M urea or 1% sodium dodecyl sulfate under the same conditions led to significant disassembly. The data suggest that both free SH groups and denaturing conditions are required for thermal disassembly to occur. Chymotrypsin, another multichain protein linked by disulfides, also exhibits disassembly in detergent at 100 °C. Studies with labeled IgG antibodies to the $Rh_0(D)$ antigen on red cell membranes indicate that membranes increase IgG disassembly during heat treatment in the presence of detergent. Thiol–disulfide interchange may be a general phenomenon with all proteins heated to 100 °C in the presence of free sulfhydryls and detergent but of major structural consequence only for multichain proteins held together by disulfide bonds.

During studies on red cell membranes with bound radiolabeled antibody, disturbances to the interchain covalent structure of immunoglobulin G (IgG)[1] were detected after treatment of sodium dodecyl sulfate solubilized membranes at 100 °C. Heat treatment is a conventional sample preparation procedure prior to sodium dodecyl sulfate–polyacrylamide gel electrophoresis of red cell membranes which serves to inactivate any leucocyte-derived proteases present.

IgG proteins are multichain macromolecules stabilized by chain complementarity and disulfide bridges (Bigelow, 1974). Like some other plasma proteins, they show a tendency to aggregate and with most commercial preparations a varying amount of dimerized or higher aggregates will occur (Finlayson et al., 1971). This lateral association, at least when heat induced, is thought to be brought about by a sulfhydryl-initiated disulfide interchange (Jensen, 1959).

This report shows that IgG chain disassembly occurs in heated sodium dodecyl sulfate solutions and is mediated by thiol–disulfide interchange. Some characteristics of this process in multichain proteins and the role of membranes are presented.

Materials and Methods

Materials. Normal human IgG which contained only intact IgG and less than 5% dimer was obtained from Miles laboratories (Elkhart, Ind.) or isolated from serum by DEAE-cellulose ion-exchange chromatography in 0.0175 M sodium phosphate buffer (pH 6.3). Chymotrypsin of the highest commercial grade was obtained from Worthington (Freehold, N.J.). Ultrapure urea was a product of Schwarz/Mann (Orangeburg, N.Y.). Glutathione and sodium dodecyl sulfate were obtained from Sigma (St. Louis, Mo.) and used without additional purification. Chemicals for electrophoresis were obtained from Bio-Rad Laboratories (Richmond, Calif.). Iodoacetamide was purchased from Aldrich (Milwaukee, Wis.) and recrystallized from hot water. ^{125}I was from New England Nuclear (Boston, Mass.).

The normal IgM (93% pure) and normal IgA (74% pure) used in these studies were provided by the American Red Cross.[2]

Methods. Electrophoretic procedures were based on the method of Fairbanks et al. (1971). Human Rh-positive red cells were freshly drawn and used in antibody binding studies according to methods previously published (Victoria et al., 1975). ^{125}I-labeled anti-$Rh_0(D)$ IgG was prepared as previously described (Masouredis et al., 1967). The preparation of ghosts from white-cell-free red cell suspensions was based on the method of Dodge et al. (1963).

Heat treatment, as the term is used in this paper, refers to exposure of electrophoresis sample solutions containing 1 mg of protein per mL to 100 °C (boiling water bath) for 10 min. Electrophoresis sample solutions contained, in addition to protein, 1% (w/v) sodium dodecyl sulfate, 10 mM Tris-HCl, 1 mM EDTA, 15% (w/v) sucrose, and 10 μg/mL Pyronin Y. The pH was 8.0. When reductant was used this was 0.6% (w/v) DTT. In separate experiments (data not shown), it was found that omission of any or all reagents except sodium dodecyl sulfate had no influence on the experimental outcome of heat treatment.

Results

Time Dependence. Exposure of IgG in sodium dodecyl sulfate to 100 °C resulted in the complete reduction of IgG to

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[1] Abbreviations used: IgG, immunoglobulin G; IgM, immunoglobulin M; IgA, immunoglobulin A; DEAE-cellulose, diethylaminoethylcellulose; DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; cmc, critical micelle concentration; BNS, 0.015 M sodium phosphate–0.15 M NaCl (pH 6.5); Tris, tris(hydroxymethyl)aminomethane.

[2] These materials were provided by the American Red Cross National Fractionation Center with the partial support of the National Institutes of Health Grant No. HL 13881.

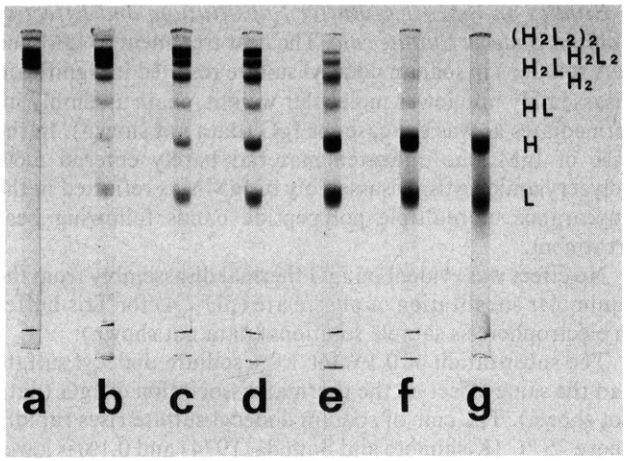

FIGURE 1: Time course of thermal disassembly of IgG in sodium dodecyl sulfate. Heat treatment was carried out as described in Methods. At the indicated times, aliquots were withdrawn and chilled in ice. After 2 h, samples containing 20 μg of IgG corresponding to each time point were run in 1% sodium dodecyl sulfate–8 M urea–5.6% polyacrylamide gels containing a monomer-to-cross-linker ratio of 20:1 at 6 mA per gel. Electrophoretic migration was toward the anode. Staining was with Coomassie blue (Korn and Wright, 1973): (a) 0; (b) 2 min; (c) 5 min; (d) 10 min; (e) 30 min; (f) 1 h; (g) 2 h.

its constituent heavy and light chains after 2 h (Figure 1). This process has been termed disassembly. No detectable disassembly occurred in samples kept at room temperature (23 °C). All of the usually recognized stages of interchain covalent assembly (H_2L_2, H_2L, H_2, HL, H, and L) appeared during this process. Identification was based on molecular weight determinations carried out with protein standards. Dimerized IgG was present in the starting material and it also was reduced. The reduction sequence appeared to proceed from higher molecular weight to lower molecular weight species with $(H_2L_2)_2$ disappearing first and HL disappearing last.

Effect of Sulfhydryl-Related Treatments. Heat treatments in the presence of iodoacetamide markedly inhibited IgG disassembly (Figure 2b vs. Figure 1d). The role of thiol–disulfide interchange was explored by utilizing at 23 °C in nondetergent solutions the interchange-promoting glutathione couple introduced by Saxena and Wetlaufer (1970) and used to study the in vitro covalent assembly of IgG by Petersen and Dorrington (1974). Presence of 10^{-4} M oxidized–10^{-3} M reduced glutathione did not lead to substantial disassembly of IgG (Figure 2c). The identical treatment, however, carried out in the presence of denaturant, either 6 M urea or 1% (w/v) sodium dodecyl sulfate, markedly promoted it (Figures 2d and 2e). At lower glutathione couple concentrations no disassembly was evident, whereas at the higher 10^{-3} M GSSG–10^{-2} M GSH concentration the IgG, even in the absence of denaturant, showed disassembly (data not shown). Although electrophoretic analyses of unreduced proteins are severely limited (Fish, 1974), it was possible to discern some differences between the action of sodium dodecyl sulfate and urea. Sodium dodecyl sulfate gave rise to discrete electrophoretic bands in addition to intact IgG corresponding to H_2L, H_2, H, and L stages (Figure 2e). With urea no free heavy chains were detected (Figure 2d). Neither denaturant resulted in the formation of HL species. In the case of urea, the electrophoretic band pattern was not so discrete as with sodium dodecyl sulfate and new bands appeared between H_2 and H_2L. The results obtained with urea may reflect carbamoylation by cyanate ions generated in the urea solution (Gerding et al., 1971).

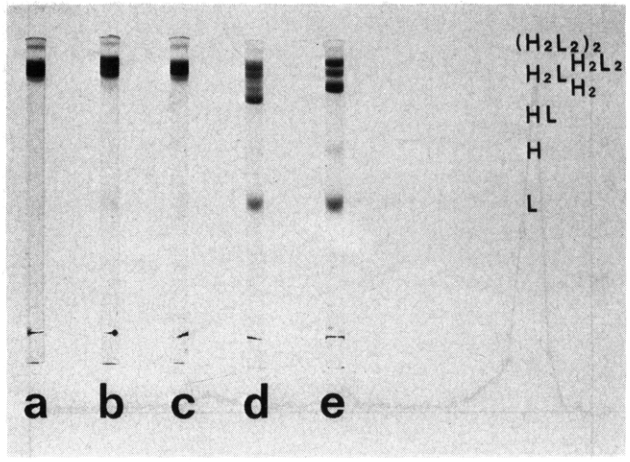

FIGURE 2: Effect of sulfhydryl-related treatments on the multichain structural stability of IgG. (a) Control. Not heat treated. IgG (2 mg/mL) incubated 30 min in water and then made up in electrophoresis sample solution to final concentrations given in Methods. (b) Heat treatment in the presence of iodoacetamide. IgG heat treated as described in Methods but 250 mM iodoacetamide included in electrophoresis sample solution. (c) Room temperature incubation with glutathione couple under nondenaturing conditions. IgG (2 mg/mL) incubated with 10^{-3} M GSH–10^{-4} M GSSG for 30 min following which the solution was made up in electrophoresis sample solution as in a. Not heat treated. (d) Incubation with glutathione couple plus urea. Conditions as in c, but 6 M urea was included in solution containing the glutathione couple. Not heat treated. (e) Incubation with glutathione couple plus sodium dodecyl sulfate. Conditions as in c, but 1% sodium dodecyl sulfate was included in glutathione couple solution. Not heat treated. All gels contained 20 μg of protein. Electrophoretic conditions as in Figure 1.

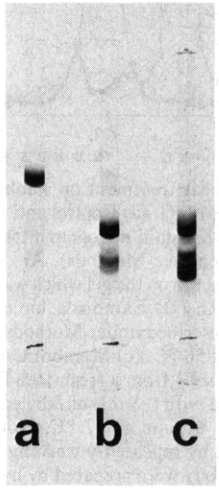

FIGURE 3: Effect of heat treatment on chymotrypsin in sodium dodecyl sulfate solutions. (a) Control. Not heat treated. Chymotrypsin made up at 1 mg/mL in electrophoresis sample solution (see Methods). (b) Heat treated. Same as a, but heat treated as described under Methods. (c) Dithiothreitol reduced. Same as a, but electrophoresis sample solution contained 0.6% (w/v) DTT. Electrophoretic conditions as in Figure 1 but in gels that were 10.0% polyacrylamide.

Studies on Heat-Treated Chymotrypsin in the Presence of Sodium Dodecyl Sulfate. Electrophoretic patterns from chymotrypsin solutions in sodium dodecyl sulfate made up at 23 °C indicated predominantly a single component (Figure 3a). Heat-treated samples, however, showed significant reduction of the enzyme to its constituent chains (Figure 3b; the smallest chain, about 1500 molecular weight, was poorly fixed and was lost from the gels during diffusion destaining). Thermal dissociation of chymotrypsin was blocked by iodoacetamide (data not shown). The heat-treated chymotrypsin

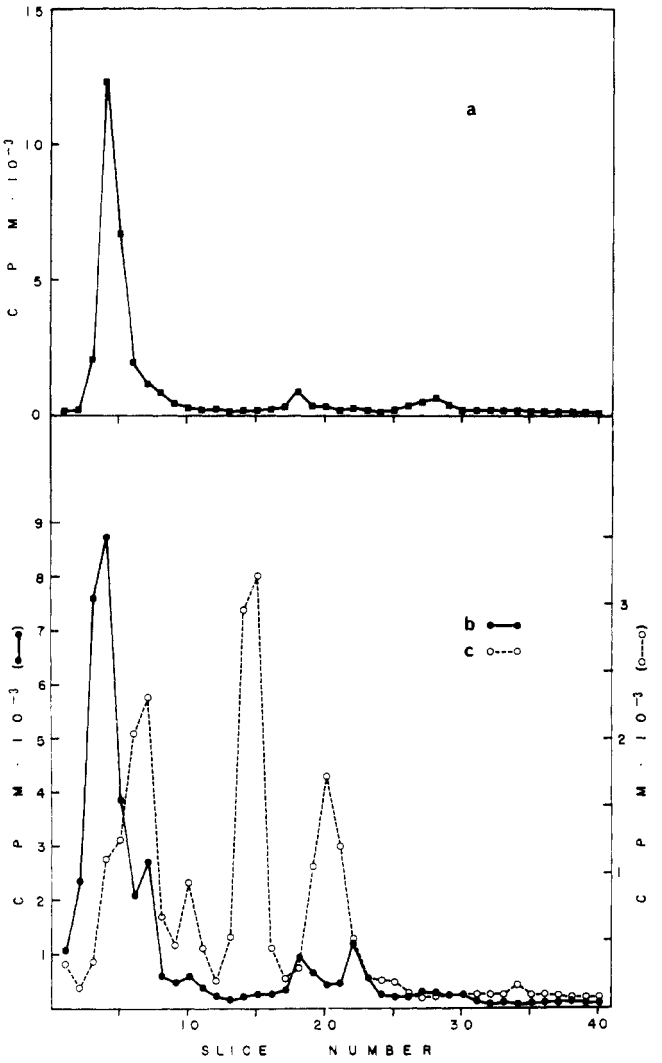

FIGURE 4: Effect of heat treatment on soluble and membrane-bound ^{125}I-labeled anti-Rh$_0$(D) IgG. (a) Control anti Rh$_0$(D). Not heat treated. Anti-Rh$_0$(D) (1 μg of Kjeldahl nitrogen/mL) was made up in electrophoresis sample solution (see Methods). An amount corresponding to approximately 20 ng was run on the gel which was sliced at 2-mm intervals. (b) Heat-treated anti-Rh$_0$(D). Same as a, but electrophoresis sample solution was heated as described under Methods (10 min at 100 °C) plus an additional 30 min at 56 °C. (c) Membrane-bound, heat-treated anti-Rh$_0$(D). Aliquots (0.1 mL) from a fresh 10% BNS-washed red cell suspension were incubated with 0.5 mL of labeled antibody (about 1 μg of Kjeldahl nitrogen) for 60 min at 37 °C. Nonimmunologically bound antibody was removed by repeatedly washing in BNS. Red cell ghosts containing bound antibody were prepared by hypotonic lysis as described under Methods. These were solubilized in electrophoresis sample solution and heat treated as described under b. The amount applied to the gel corresponded to 25 μg of membrane protein. Electrophoretic migration was toward the right (anode).

pattern resembled that seen after reduction with DTT (Figure 3c).

Effect of Heat Treatment on Labeled Anti-Rh$_0$(D) IgG Bound to Red Cell Membranes after Solubilization in Sodium Dodecyl Sulfate. Solutions of heat-treated ^{125}I-labeled anti-Rh$_0$(D) alone yielded the radioelectrophoretic pattern illustrated in Figure 4b. Although partial disassembly is evident in comparison with room temperature anti-Rh$_0$(D) (Figure 4a), it is far less than the extent evident when antibody bound to membranes undergoes the same treatment (Figure 4c). The pattern of unheated membrane-bound antibody was essentially the same as Figure 4a.

Studies on IgM, IgA, Buffer Substitution, and Effect of Sodium Dodecyl Sulfate cmc. The heat treatment of IgM and IgA solutions in sodium dodecyl sulfate resulted in significant disassembly into lower molecular weight, chain assembly intermediates as was the case for IgG (data not shown). In the case of IgM, the unheated material barely entered 5.6% polyacrylamide gels. Disassembly of IgM was reflected in the appearance of multiple polypeptide bands following heat treatment.

No effect was evident on IgG thermal disassembly from the equimolar substitution of phosphate (pH 7.4) for Tris buffer in electrophoresis sample solutions (data not shown).

The substitution of 0.1% for 1.0% sodium dodecyl sulfate had the same effect on the thermal dissociation of IgG (data not shown). The cmc of sodium dodecyl sulfate rises rapidly above 25 °C (Kishimoto and Sumida, 1974) and 0.1% is lower than most reported sodium dodecyl sulfate cmc values so that heat treatment in 0.1% sodium dodecyl sulfate probably occurred in the absence of micellar sodium dodecyl sulfate.

Discussion

The disulfide bond is the most labile covalent linkage commonly found in proteins (Wall, 1971) and multiple mechanisms exist for its scission (Parker and Kharasch, 1959). In biological systems reduction probably occurs predominantly through exchange reactions. The occurrence of disulfide interchange during thermal denaturation of proteins has long been known (reviewed by Jensen, 1959). These early studies showed that, particularly in plasma proteins, heating led to aggregation and, with increasing heat, to precipitation.

The presence of a strong surfactant such as sodium dodecyl sulfate allows for temperature treatments to be extended into ranges where precipitation would normally occur.[3] Heat denaturation in sodium dodecyl sulfate has not received much attention, understandably, since the capacity of either heat or sodium dodecyl sulfate to bring about denaturation is well known and, indeed, each separate process has been investigated extensively (Joly, 1965; Tanford, 1968; Steinhardt and Reynolds, 1969). However, Satake and Yang (1975) have shown that, in polylysine solutions made up in 0.5% sodium dodecyl sulfate, a great increase in helicity occurred as the temperature was raised from 25 to 70 °C. In addition, it was recently reported that heat treatment up to 100 °C promoted helix formation in sodium dodecyl sulfate solutions of *E. coli* membrane proteins (Nakamura and Mizushima, 1976).

The ability of strong anionic surfactants to cause conformational changes is well established (Steinhardt and Reynolds, 1969) and, recently, Jirgensons (1973) described an increase in helix content for IgG in sodium dodecyl sulfate solutions at ambient temperature. Thus, thermal denaturation in the presence of sodium dodecyl sulfate should enhance the helical content of IgG and bring about the necessary proximity of free sulfhydryls to disulfides required for interchange to occur.

The use of glutathione couple to regenerate disulfide-reduced proteins was introduced by Saxena and Wetlaufer (1970) and was recently applied (in addition to air oxidation) by Petersen and Dorrington (1974) to study the interchain covalent assembly of IgG starting from free chain constituents. An independent investigation (Sears et al., 1975) has used trace-metal-catalyzed air oxidation throughout to also study the same process. The findings presented in Figure 1 describing the sequence of disassembly bear a remarkable similarity, if

[3] IgG in 1% solutions of the mild detergents sodium deoxycholate (anionic) and Triton X-100 (non-ionic) coagulated upon heat treatment.

read in reverse order, to results of sodium dodecyl sulfate gel electrophoresis illustrating the progression of chain *assembly* seen with IgG1 (Figure 2 of Petersen and Dorrington, 1974; Figure 1 of Sears et al., 1975). Normal IgG from human serum, such as used in this investigation, is over two-thirds IgG1 (Nisonoff et al., 1975). An adequate assessment of the relevance of the pathway of IgG *disassembly* in heat-treated sodium dodecyl sulfate solutions toward an understanding of the converse pathway must await an investigation focusing on the kinetics and pathway of reduction.

Incubations of IgG in nondetergent solutions with the appropriate concentrations of glutathione couple did not lead to significant chain disassembly. This is consistent with the important role of noncovalent interactions in maintaining the quaternary structure of IgG (Bigelow, 1974; Cathou and Dorrington, 1975; Venyaminov et al., 1976). The inclusion of urea or sodium dodecyl sulfate in those solutions, however, facilitated the conversion of H_2L_2 to lower molecular weight intermediates. Thus, disulfide interchange under chemical or thermal denaturing conditions can lead to IgG breakdown. Although the manner of IgG disassembly probed in this investigation occurred under entirely unphysiological conditions, the spontaneous dissociation under mild conditions of an IgG3 paraprotein through disulfide interchange has been recently reported (Bata and Francoeur, 1975).

IgG has a low yet measurable thiol content, approximately 0.2 mol of SH/mol of IgG (Buchwald and Connell, 1974). The importance of SH group participation in IgG thermal disassembly in sodium dodecyl sulfate is indicated by the inhibition of this process by the alkylating reagent iodoacetamide. This was previously recognized by Virella and Parkhouse (1973) who included iodoacetamide in their electrophoresis sample solutions to protect the integrity of IgG during exposure to 100 °C for 2 min.

Deutsch (1976) recently undertook a systematic study involving six standard proteins (all single chain polypeptides or composed of noncovalently held subunits dissociable in sodium dodecyl sulfate) on the effect of prolonged 100 °C treatment in sodium dodecyl sulfate and mercaptoethanol upon sodium dodecyl sulfate gel electrophoresis patterns. All proteins withstood up to a 30-min treatment and most up to 3 h. Since disassembly of IgG is apparent within 1 min of heat treatment, it seemed clear that his findings could not be extended to multichain proteins stabilized by interchain disulfide bonds and whose full covalent structure can only be studied in the absence of disulfide reduction. The latter type of proteins are relatively rare (e.g., the immunoglobulins, insulin, α-chymotrypsin, and others compiled by Kleine, 1972).

α-Chymotrypsin which consists of three polypeptide chains linked by two interchain disulfide bonds (Blow, 1971) was used, like IgG, to study thermal chain disassembly in sodium dodecyl sulfate solutions. Heat treatment of chymotrypsin in sodium dodecyl sulfate resulted in chain disassembly mediated by disulfide interchange. Since the process requires the participation of free sulfhydryls, the behavior of chymotrypsin appeared to contradict this. Chymotrypsin contains ten cysteines but all participate in the formation of five disulfide bonds. The results appear explainable, however, on the basis of the level of SH-bearing contaminants present in the enzyme preparation which, although of the highest commercial grade, was not free of autolysis products and low molecular weight contaminants (evident in Figure 3a).

The results obtained with red cell membranes sensitized with radiolabeled anti-$Rh_0(D)$ indicate that membranes are not passive participants in the thermal disassembly of IgG in detergent solutions. Membrane-bound IgG exhibited greater reduction than soluble antibody after heat treatment. The mechanism of membrane involvement is unknown but it appears likely that, in spite of their low thiol content (Steck and Fox, 1972), they provide a reservoir of SH groups to participate in exchange processes. The participation of membrane polypeptides in IgG disassembly suggests that they themselves are actively involved in disulfide interchange.

The data obtained suggest that disulfide interchange occurring in sulfhydryl-containing heated sodium dodecyl sulfate solutions may be a general property of all cystine-containing proteins. Lack of attention to it is probably due to: most proteins not consisting of multiple chains stabilized by disulfides and thus incapable of reflecting structural consequences in sodium dodecyl sulfate gel electrophoresis; the use of disulfide reduction in conjunction with sodium dodecyl sulfate gel electrophoresis; and most proteins not being subjected to heat treatment prior to electrophoresis. It is likely, however, that the universality of the process may make it amenable to exploitation in the future.

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Couplings between the Sites for Methionine and Adenosine 5′-Triphosphate in the Amino Acid Activation Reaction Catalyzed by Trypsin-Modified Methionyl-Transfer RNA Synthetase from *Escherichia coli*†

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ABSTRACT: Couplings (i.e., the changes in dissociation constant of one ligand upon ligation of another ligand) within the adenylating site of trypsin-modified methionyl-tRNA synthetase have been systematically analyzed with the use of analogues (or substructures) of methionine and ATP-Mg^{2+} molecules. Once bound to the enzyme, the specific amino acid side chain can couple as well with the binding of adenosine as with the binding of pyrophosphate. The primary coupling involving the subsites for adenosine and methioninol (an analogue of methionine lacking the carboxylate group) corresponds to a free energy of interaction, $\Delta G = 0.8$ kcal, which is enhanced by the carboxyl function of the amino acid or the α-phosphoryl group of 5′-adenosine nucleotides. Upon introduction of the carboxylate, the gain in Gibbs free energy of interaction for adenosine binding is equal to 0.3 kcal. Reciprocally the association of a 5′-adenosine nucleotide (ATP, ADP, or AMP) to the enzyme-bound methioninol is improved with respect to adenosine by values ranging from 1 to 2.2 kcal depending on the nucleotide considered and the presence of magnesium. These observations indicate that manifestation of major synergistic couplings requires neutralization of a cationic locus at the surface of the enzyme. Direct evidence that the carboxylate and α-phosphate groups are directed toward such a common enzymic locus is provided by the antagonistic couplings exerted on methionine binding either by ADP or AMP. Magnesium is involved in the couplings only when polyphosphoryl groups are engaged within the enzyme site. The effect of magnesium is to increase the free energies of couplings between ligands, one of which occupies the β and γ subsites of the ATP site. For instance, upon introduction of the divalent ion, the free-energy gain on methionine binding (in the presence of adenosine and pyrophosphate) rises up to 3.4 kcal. Thus, arising first of all from the specific binding of the amino acid is a series of self-amplifying coupled binding processes which take advantage of magnesium, the cofactor of catalysis. The resulting free energy which is no longer observed at the level of the methionine/ATP-Mg^{2+} coupling (prior to the reaction) is assumed to overcome the geometric and entropic requirements for aminoacyl adenylate synthesis.

Prior to their participation in polypeptide bond formation on ribosomes, tRNAs are specifically aminoacylated by aminoacyl-tRNA synthetases. The enzymatic aminoacylation of tRNA involves the activation of the amino acid through adenylate formation, followed by transfer of the aminoacyl moiety to a specific tRNA (for a review, see Kisselev and Favorova, 1974, and references therein). Several mechanisms have been proposed to account for the low rate of overall error in protein synthesis (Loftfield and Vanderjagt, 1972). The specificity of the aminoacylation reaction may be improved through "kinetical proofreading" (Hopfield, 1974; Ninio, 1975; Hopfield et al., 1976), "hydrolytic editing" (Fersht and Kaethner, 1976), or "chemical proofreading" (von der Haar and Cramer, 1976) of misacylated tRNAs on the synthetase itself. Such mechanisms may be of the utmost importance in order to discriminate against misactivation of amino acids which are isosteric with the correct one. While several examples of misactivation of amino acids by aminoacyl-tRNA synthetases have been discovered and are well documented (Baldwin and Berg, 1966), it is nevertheless clear that in most cases activation of the correct amino acid remains a key feature of the overall specificity of the aminoacylation reaction.

The coupling between ligands (i.e., the change in the dissociation constant of one ligand upon binding of another ligand) which has been observed within the adenylating site of several aminoacyl-tRNA synthetases (Kosakowski and Holler, 1973; Fayat and Waller, 1974; Blanquet et al., 1975b; Holler et al., 1975) may well be an important factor contributing to the specificity of the amino acid activation reaction.

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