Gerhart, J. C., and Pardee, A. B. (1962), *J. Biol. Chem. 237,* 891.
Gerhart, J. C., and Schachman, H. K. (1965), *Biochemistry 4,* 1054.
Gerhart, J. C., and Schachman, H. K. (1968), *Biochemistry 7,* 538.
Goa, J. (1953), *Scand. J. Clin. Lab. Invest. 5,* 218.
Gray, C. W., Chamberlin, M. J., and Gray, P. M. (1973), *J. Biol. Chem. 248,* 6071.
Griffin, J. H., Rosenbusch, J. P., Blout, E. R., and Weber, K. K. (1973), *J. Biol. Chem. 248,* 5057.
Griffin, J. H., Rosenbusch, J. P., Weber, K. K., and Blout, E. R. (1972), *J. Biol. Chem. 247,* 6482.
Hill, E., Tsernoglou, D., Webb, L., and Banaszak, L. J. (1972), *J. Mol. Biol. 72,* 577.
Hinz, H. J., Shiao, D. D., and Sturtevant, J. M. (1971), *Biochemistry 10,* 1347.
Ho, H. C., and Wang, J. H. (1973), *Biochemistry 12,* 4750.
Jacobson, G. R., and Stark, G. (1973), *Enzymes, 3rd Ed. 9,* 226.
Jänig, G. R., Gerber, G., Ruckpaul, K., Rapaport, S., and Jung, R. (1970), *Eur. J. Biochem. 17,* 441.
Kauzmann, W. (1959), *Advan. Protein Chem. 14,* 1.
Kirschner, M. W., and Schachman, H. K. (1971), *Biochemistry 10,* 1900.
Kleppe, K., (1966), *Biochim. Biophys. Acta 122,* 450.
London, R. E., and Schmidt, P. G. (1972), *Biochemistry 11,* 3136.
Markus, G., McClintock, D. K., and Bussel, J. B. (1971), *J. Biol. Chem. 246,* 762.
Matsumoto, S., and Hammes, G. G. (1973), *Biochemistry 12,* 1388.
McClintock, D. K., and Markus, G. (1968), *J. Biol. Chem. 243,* 2855.
McClintock, D. K., and Markus, M. (1969), *J. Biol. Chem. 244,* 36.
Meadows, D. H., and Jardetsky, O. (1968), *Proc. Nat. Acad. Sci. U. S. 61,* 406.
Nelbach, M. E., Pigiet, V. P., Gerhart, J. C., and Schachman, H. K. (1972), *Biochemistry 11,* 315.
Pigiet, V. (1971), Ph.D. Thesis, University of California, Berkeley.
Richards, F. M., Wyckoff, H. W., and Allewell, N. (1970), *in* the Neurosciences Second Study Program, Schmitt, F. O., Ed., New York, N. Y., Rockefeller Press, p 901.
Ross, P. P., and Ginsberg, A. (1969), *Biochemistry 8,* 4690.
Rosenbusch, J. P., and Weber, K. (1971), *J. Biol. Chem. 246,* 1644.
Sturtevant, J. M. (1969), *Fractions 1,* 1.
Sturtevant, J. M., and Lyons, P. A. (1969), *J. Chem. Thermodyn. 1,* 201.
Tondre, C., and Hammes, G. G. (1974), *Biochemistry 13,* 3131.
Velick, S. F., Baggott, J. P., and Sturtevant, J. M. (1971), *Biochemistry 10,* 779.
Weber, K. (1968), *Nature (London) 218,* 1116.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Winlund, C. C., and Chamberlin, M. J. (1970), *Biochem. Biophys. Res. Commun., 40,* 43.

Properties of the Highly Reactive SH Groups of Phosphorylase *b*†

Tore Sanner* and Lajos Tron‡

ABSTRACT: The reaction of rabbit muscle phosphorylase *b* with 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2) has been studied with stopped-flow spectrophotometry. Two highly reactive sulfhydryl groups per dimer reacted with Nbs_2 within a few seconds, while the remaining SH groups needed several minutes and hours. Decomposition of the time curve revealed that the highly reactive SH groups can be divided into two subclasses: a fast type which reacted with a rate constant of 3×10^3 M^{-1} sec^{-1} and a more slowly reacting type disappearing with a rate constant of 0.3×10^3 M^{-1} sec^{-1}. The reactivity of the slowly reacting type increased by a factor of about 2 in the presence of 1 mM AMP. Concurrently, the ratio between the fast reacting and the more slowly reacting subclasses decreased from 5.3 to 1.0. The AMP effect was greatly enhanced by glucose 1-phosphate. This enhancement was abolished in the presence of ATP. The finding that the ratio between the number of SH groups in the two subclasses of the highly reactive SH groups changed upon addition of ligand molecules indicates that the two subclasses reflect the different reactivities of the SH groups when the enzyme is present in different conformational states. It is suggested that the highly reactive SH group measured belong to the peptide: Gly-Cys-Arg-Asp.

Rabbit muscle phosphorylase *b* (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) possesses at least three types of SH-groups with widely different reactivity (Damjanovich and Kleppe, 1966; Gold, 1968; Kastenschmidt *et al.*, 1968a; Kleppe and Damjanovich, 1969; Avramovic-Zikic *et al.*, 1970; Zarkadas *et al.*, 1970; Hasinoff *et al.*, 1971). Two to four SH groups react very rapidly with most sulfhydryl reagents. Approximately four SH groups show a moderate reactivity. Finally, the enzyme has 10–12 SH groups which react very slowly with most re-

†From Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, Oslo 3, Norway. *Received August 19, 1974.* This work was supported by a fellowship to L.T. from the Norwegian Government.

‡Permanent address: Department of Biophysics, Medical University of Debrecen, Hungary.

agents unless sodium lauryl sulfate, urea, or other unfolding agents are present. Blocking of the moderately reactive groups results in complete loss of enzyme activity and dissociation of the enzyme into subunits (Madsen and Cori, 1956; Kastenschmidt *et al.*, 1968a; Kleppe and Damjanovich, 1969). The reactivity of these groups is reduced by the presence of the activator AMP (Jokai *et al.*, 1965; Kleppe and Damjanovich, 1969; Avramovic-Zikic *et al.*, 1970).

Little is known about the role of the highly reactive sulfhydryl groups. These SH groups react nearly 10,000 times faster with *p*-chloromercuribenzoate than the moderately reactive SH groups (Hasinoff *et al.*, 1971). Gold (1968) has obtained evidence that AMP reduces the reactivity of the highly reactive sulfhydryl groups in a similar manner as it reduces the reactivity of the moderately reactive SH groups. Although these SH groups are not directly involved in the catalysis, blocking of the highly reactive SH groups seems to affect the structure of the enzyme. Thus, Damjanovich *et al.* (1967) found that blocking of these SH groups reduced the sensitivity of the remaining SH groups to oxidation.

In the present paper, the reaction of the highly reactive sulfhydryl groups of phosphorylase *b* with 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2)[1] have been studied under different conditions. Nbs_2 was chosen as it reacts specifically with SH groups and the reaction can easily be followed spectrophotometrically (Ellman, 1959). Moreover, the effect of blocking of the highly reactive SH groups on the kinetic parameters of the enzyme is studied.

Experimental Procedures

Phosphorylase b was prepared from rabbit muscle according to Fischer and Krebs (1958). It was recrystallized five times and subsequently lyophilized from a solution containing 50 mM Tris-chloride buffer (pH 6.8), 0.33 mM cysteine, and 1 mM AMP. Immediately before use, the enzyme (20–30 mg of protein) was dissolved in 1 ml of distilled water containing 1 mM EDTA and 1 mM cysteine and passed though a Sephadex G-25 column (1.5 × 12 cm) to remove cysteine and AMP. The column was equilibrated with 50 mM Tris-chloride buffer (pH 6.8) containing 1 mM EDTA. The buffer used for equilibration and elution of the column was bubbled with nitrogen. The eluted enzyme was kept in a small glass tube sealed with parafilm to avoid exposure to air. The column chromatography was carried out at room temperature and the enzyme was kept at this temperature until use. The ratio of absorbance at 260 and 280 nm was 0.56. Protein concentrations were calculated from the absorbance at 280 nm using a value for $E_{1\,cm}(1\%)$ of 11.9 (Appleman *et al.*, 1963). The concentrations are expressed as molar concentrations of the dimer (MW 185,000) (Seery *et al.*, 1967).

Enzyme Assay. The activity of phosphorylase *b* was determined from the amount of P_i liberated from glucose 1-phosphate. The standard assay mixture contained 16 mM glucose 1-phosphate, 1 mM AMP, 1% glycogen, 50 mM Tris-chloride buffer, 1 mM EDTA, and approximately 10^{-7} M phosphorylase *b* in a total volume of 0.4 ml. The pH of all solutions was adjusted to pH 6.8 and the temperature during incubation was 30°. After incubation of the mixture for 10 min the reaction was stopped by adding 2.6 ml of 5% trichloracetic acid. The amount of P_i liberated was determined according to Taussky and Shorr (1953).

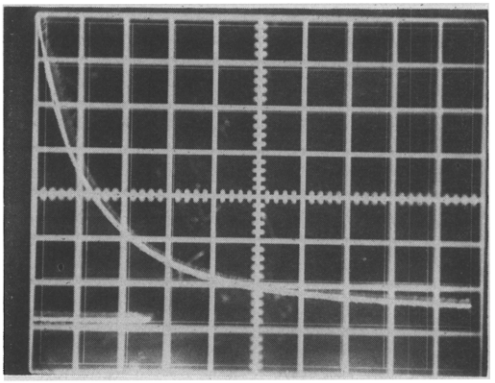

FIGURE 1: Change in transmission as a function of time after mixing of phosphorylase *b* and Nbs_2 in the stopped-flow spectrophotometer. The two syringes were filled with phosphorylase *b* (2.0 μM) and Nbs_2 (1 mM), respectively. All solutions were made up in 50 mM Tris-chloride buffer (pH 6.8) containing 1 mM EDTA. Scanning rate, 0.5 sec/large division; sensitivity, 50 mV/cm; total signal, 5 V. The trace in the lower left corner represents the transmission measured after 20 sec.

Reaction with Nbs_2. The reaction of Nbs_2 with the SH groups was carried out according to Ellman (1959) by measuring increase in absorption at 412 nm. A Durrum stopped-flow spectrophotometer, Model 110, with a cuvet with 20-mm light path was used in the kinetic experiments. All experiments were carried out at 25° in 50 mM Tris-chloride buffer (pH 6.8) containing 1 mM EDTA. For calculation of the number of SH groups reacted, the molar extinction coefficient for thio-2-nitrobenzoic acid of 13,600 was used (Ellman, 1959).

In the kinetic experiments with the stopped-flow spectrophotometer, the change in transmission was displayed on a Tektronix storage oscilloscope. Photographs of the curves were taken with a Polaroid camera. The changes in transmission were measured and converted to absorption by the use of a Hewlett Packard calculator combined with a *X–Y* plotter, as previously described (Sanner, 1971).

Materials. Glucose-1-P, rabbit liver glycogen, AMP, ATP, EDTA, and Nbs_2 were obtained from Sigma Chem. Co., St. Louis, Mo.

Results

Figure 1 shows the trace obtained on the oscilloscope upon mixing of phosphorylase *b* with Nbs_2 in the stopped-flow spectrophotometer. A rapid decrease in the transmission occurred during the first second and then the curve leveled off. The lower trace shows the transmission 20 sec after mixing. Since under the present experimental conditions less than 0.4% of the moderately reactive sulfhydryl groups have reacted during the first 20 sec, no corrections were made for interaction of Nbs_2 with these groups and the absorption measured after 20 sec was used as final absorption for calculation of the total number of highly reactive sulfhydryl groups.

On the basis of the oscilloscope traces the change in absorption during the reaction was calculated. A typical result is shown in Figure 2A. From the total increase in absorption the number of highly reactive SH groups was calculated. In the different experiments the number varied from 2.0 to 2.3.

In order to determine the rate constant of the reaction, the number of unreacted sulfhydryl groups in per cent was

[1] Abbreviations used are: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); glucose-1-P, α-D-glucose 1-phosphate.

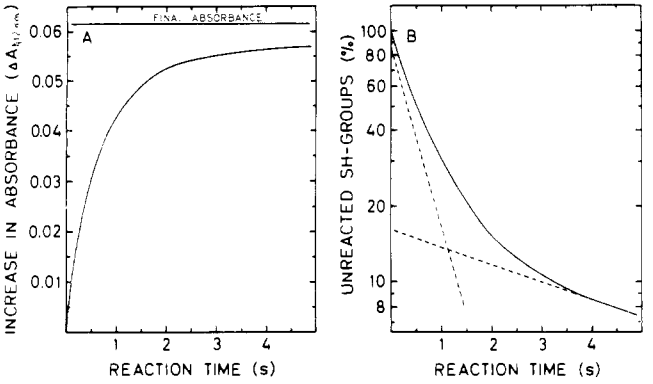

FIGURE 2: Reaction of Nbs_2 with the highly reactive SH groups of phosphorylase *b*. (A) Increase in absorption as a function of reaction time. The absorption has been calculated from the data in Figure 1 and represents the absorption measured in a 20-mm cuvet. The final absorption was obtained 20 sec after the initiation of the reaction. (B) Unreacted sulfhydryl groups as a function of reaction time. The number of unreacted SH groups in per cent (calculated from the data in part A) has been plotted in a logarithmic scale *vs.* the reaction time. The dashed lines show the decomposition of the curve into two first-order reactions.

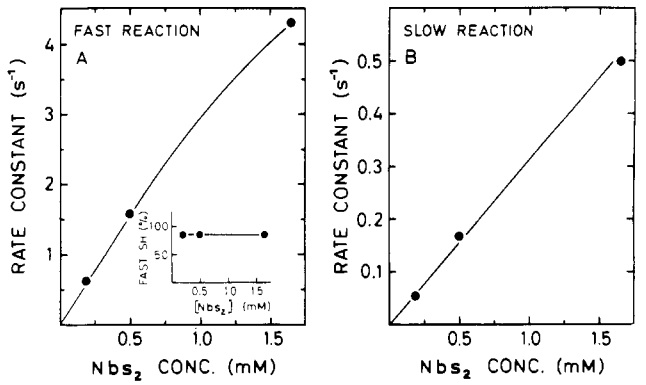

FIGURE 3: Effect of Nbs_2 concentration on the reaction rate of the highly reactive SH groups of phosphorylase *b*. (A) Apparent first-order rate constant for the fast reacting SH groups as a function of the Nbs_2 concentration. The inserted figure shows the number of fast reacting SH groups in percentage of the total number of highly reactive sulfhydryl groups as a function of the Nbs_2 concentration. The number of fast and slowly reacting SH groups was determined as described in the text. (B) Apparent first-order rate constant for the slowly reacting SH groups as a function of the Nbs_2 concentration. The final concentration of phosphorylase *b* was 1.0 μM and the final concentration of Nbs_2 as indicated. Otherwise conditions as in Figure 1.

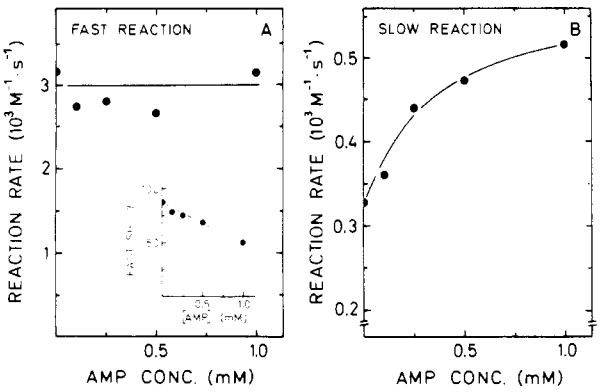

FIGURE 4: Effect of AMP concentration on the rate of the reaction between the highly reactive sulfhydryl groups of phosphorylase *b* and Nbs_2. (A) Second-order rate constant for the fast reacting SH groups as a function of the AMP concentration. The inserted figure shows the number of fast reacting SH groups in percentage of the total number of highly reactive SH groups as a function of the AMP concentration. (B) Second-order rate constant for the slowly reacting SH groups as a function of the AMP concentration. AMP was in all experiments premixed with the enzyme 5 min prior to the experiment. Concentrations and experimental conditions as in Figure 1.

plotted in a logarithmic scale *vs.* the reaction time (Figure 2B). It is seen that a biphasic curve was obtained. This curve was decomposed by assuming that it is composed of two first-order reactions. The decomposition was made manually, as indicated. In the present experiment 16% of the SH groups reacted more slowly than the other ones. The rate constant differed by a factor of about 10. The two reactions will in the following be referred to as the "fast reaction" and the "slow reaction" of the highly reactive SH groups. The finding of two subclasses of highly reactive sulfhydryl groups can either imply that they represent different types of SH groups in the enzyme or that the enzyme is present in different states in which the highly reactive SH groups possess different reactivity.

When the apparent first-order rate constant of the reaction was determined for different concentrations of Nbs_2 (Figure 3), the rate constant for the fast as well as for the slow reaction of the highly reactive SH groups increased nearly linearly with the Nbs_2 concentration. In separate experiments it was found that rate constant was independent of the enzyme concentration (data not presented). Thus, the interaction occurs according to bimolecular reaction kinetics. In the remaining part of the paper, the reaction rates have been expressed as the second-order rate constants. As expected the percentage of the fast reacting sulfhydryl groups is independent of the Nbs_2 concentration (insert, Figure 3A).

As pointed out above, Gold (1968) observed that AMP seemed to protect the highly reactive sulfhydryl groups against chlorodinitrobenzene. In order to study the effect of AMP on the highly reactive SH groups of phosphorylase *b* in more detail, we have repeated this experiment with Nbs_2.

The results (Figure 4A) show that the rate constant for the fast reaction is independent of the AMP concentration, while the rate constant for the slow reaction (Figure 4B) increases strongly. Concurrently the percentage of the fast reacting sulfhydryl groups decreases from 84% in the absence of AMP to about 50% in the presence of 1 mM AMP (insert Figure 4A). If the increase in the reaction rate of the slow reaction is plotted as a function of the AMP concentration in a double inverse plot, a straight line is obtained with K_{AMP} = 0.6 mM. This value is close to the dissociation constant for AMP in the absence of glucose-1-P (Kastenschmidt *et al.*, 1968b).

The present results are in contrast with those reported by Gold (1968). The discrepancies can probably be accounted for by the fact that Gold (1968) observed only one class of highly reactive sulfhydryl groups. As the fraction of the slowly reacting SH groups increases in the presence of AMP, this implies that if the experiments are carried out under conditions where one is unable to differentiate between the two subclasses an apparent decrease in the overall reaction rate will be observed.

The most interesting finding is that although the same number of SH groups was titrated in the presence of AMP, the ratio between the "fast reacting" and the more "slowly reacting" sulfhydryl groups decreased strongly. This observation indicates that the fast reacting and the slowly reacting SH groups reflect different reactivities of the highly re-

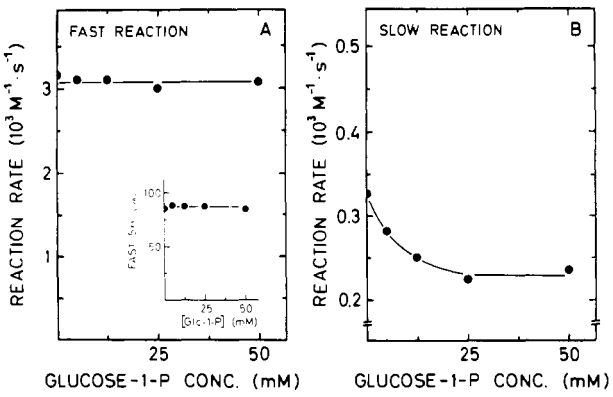

FIGURE 5: Effect of glucose-1-P concentration on the rate of the reaction between the highly reactive SH groups of phosphorylase *b* and Nbs_2. (A) Second-order rate constant for the fast reacting SH groups as a function of the glucose-1-P concentration. The inserted figure shows the number of fast reacting SH groups in percentage of the total number of highly reactive SH groups as a function of the glucose-1-P concentration. (B) Second-order rate constant for the slowly reacting SH groups as a function of the glucose-1-P concentration. Glucose-1-P was in all experiments premixed with the enzyme 5 min prior to the experiment. Concentrations and experimental conditions as in Figure 1.

active SH groups when the enzyme is present in different states. The data are consistent with the view that AMP affects the relative amount of enzyme in the two states.

The reaction rate of the sulfhydryl groups in the fast reaction subclass was independent of the presence of the substrate, glucose-1-P (Figure 5A), while the reaction rate of the slow reaction decreased (Figure 5B). If the decrease in reaction rate with substrate is plotted in a double inverse plot, a straight line with $K_{\text{glucose-1-P}} = 11$ mM is obtained.

The percentage of the two subclasses is independent of the amount of substrate present (insert Figure 5A). Moreover, the total number of highly reactive sulfhydryl groups was the same whether substrate was present or not.

In order to study whether any cooperativity could be observed between AMP and glucose-1-P, experiments were carried out in which the reaction rates were determined in the presence of a mixture of the two ligands. Table I shows that addition of glucose-1-P enhanced the AMP effect. Thus, the rate of the slow reaction increased to a greater extent when AMP and glucose-1-P was added together than in the presence of AMP alone. Likewise the ratio between the SH groups in the two subclasses decreased to a much larger extent when the mixture of the two ligands was added than in the presence of AMP alone. Interestingly, the reaction rate of the fast reaction increased in spite of the fact that none of the two ligands have any significant effect on this reaction rate when added singly.

ATP is an inhibitor of the enzymatic reaction catalyzed by phosphorylase *b* and it is assumed that it competes with the activator AMP. The reaction rates as well as the ratio between the two subclasses were affected in the same way by ATP and AMP. However, much higher concentrations of ATP are needed to achieve the effects (Table I). Interestingly, when ATP was added in the presence of AMP and glucose-1-P, ATP abolished the enhancement of the AMP effect induced by glucose-1-P.

Table II shows the effect of SH blocking on the kinetic parameters. Blocking of the two highly reactive sulfhydryl groups had no effect on V when AMP was varied. K_{AMP} increased, however, by a factor of nearly 2.5. When glucose-1-P was the variable substrate a 20% decrease in V was

Table I: Cooperative Effect of Ligands on the Highly Reactive SH Groups.[a]

Additions	Reaction Rates		Fast React. SH
	k_1 (10^3 M^{-1} sec^{-1})	k_2 (10^3 M^{-1} sec^{-1})	Slowly React. SH
None	3.2	0.33	5.3
AMP (0.5 mM)	2.7	0.47	2.2
G-1-P (5 mM)	3.2	0.28	6.7
ATP (5 mM)	2.4	0.43	3.2
AMP (0.5 mM) + G-1-P (5 mM)	4.7	0.55	0.8
AMP (0.5 mM) + G-1-P (5 mM) + ATP (5 mM)	2.6	0.54	2.3

[a] Experimental conditions as in Figure 1. k_1 and k_2 refer to the second-order rate constant of the fast and slow reaction, respectively.

Table II: Effect of SH Blocking on the Kinetic Parameters.[a]

No. of SH Groups Blocked	Variable Substrate: Glucose-1-P		Variable Substrate: AMP	
	V (%)	K_m (mM)	V (%)	K_A (mM)
0	100	11	100	0.045
2	80	11	100	0.110
3.5	40	8	70	0.090

[a] Phosphorylase *b* (2.6×10^{-6} M) was incubated with Nbs_2 (0.5×10^{-3} M) at 25° in Tris-chloride buffer (50 mM) (pH 6.8) containing 1 mM EDTA. Samples were taken after 1.5- and 25-min incubation. Determination of the absorption at 412 nm demonstrated that under the conditions used, 2 SH groups had reacted during the first 1.5 min and 3.5 after 25 min. The kinetic parameters were determined from Lineweaver–Burk plots. An AMP concentration of 1 mM was used when the concentration of glucose-1-P was varied and the glucose-1-P concentration was 16 mM when AMP was varied.

found. Part of this decrease can be accounted for by the increase observed in K_{AMP} as the experiments were carried out with 1 mM AMP. K_m for glucose-1-P was not affected by blocking of the highly reactive sulfhydryl groups. Blocking of part of the moderately reactive sulfhydryl groups resulted in a significantly decrease in V. K_{AMP} showed a small decrease compared to that observed after blocking of the highly reactive sulfhydryl groups, while K_m for the substrate decreased by about 25%.

Discussion

In the present experiments two highly reactive sulfhydryl groups in phosphorylase *b* dimers reacted with Nbs_2 within a few seconds. The SH groups could be divided into two subclasses which differed in reactivity with a factor of about 10. The finding of two highly reactive sulfhydryl

groups per dimer is in agreement with previous results obtained when the enzyme is prepared in the presence of cysteine (*e.g.*, Damjanovich and Kleppe, 1966, Birkett *et al.*, 1971). It has been shown, however, that when the enzyme is prepared in the presence of thiols which are less prone to form mixed disulfide and special care is taken to avoid oxidation four highly reactive sulfhydryl groups per dimer are present (Gold, 1968, Gold and Blackman, 1970, Zarkadas *et al.*, 1970). The peptide containing the highly reactive sulfhydryl group has been identified (Gold and Blackman 1970, Zarkadas *et al.*, 1970). By comparing the results obtained by Battell *et al.* (1968) with those of Gold and Blackman (1970) and Zarkadas *et al.* (1970) it seems likely that when the enzyme is prepared in the presence of cysteine, the highly reactive SH groups per monomer belong to the peptide Gly-Cys-Arg-Asp (peptide B in the nomenclature used by Gold and Blackman (1970)).

The reason why previous authors have not obtained evidence for any subclasses of the highly reactive SH groups in peptide B is probably due to the fact that experiments with Nbs_2 (Damjanovich and Kleppe, 1966) and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (Birkett *et al.*, 1971) were carried out manually and that *p*-chloromercuribenzoate (Hasinoff *et al.* (1971) possesses too high reactivity to be able to differentiate between the two subclasses.

The ratio between the two subclasses of the highly reactive SH groups decreased from 5.3 in the absence of added ligands to 1 in the presence of 1 mM AMP. Since this effect of AMP was independent of whether AMP was premixed with the enzyme prior to the reaction with Nbs_2 (data not presented) it follows that the changes in the ratio were very rapid compared to the rate of interaction of Nbs_2 with the SH groups of peptide B. This finding makes it unlikely that the change in the ratio between the fast and slowly reacting subclasses could be related to a dimer–tetramer equilibria. Moreover, if the change in the ratio was due to AMP induced disulfide exchange this would be expected to be a much slower process. Most likely the AMP induced change in the ratio between the two subclasses reflects different reactivities when the SH groups are present in different states.

Although it is generally agreed that blocking of the highly reactive SH groups of phosphorylase *b* does not affect the maximum rate, considerable discrepancies are reported concerning the effect on the binding of ligands. Avramovic-Zikic *et al.* (1970) were unable to observe any effect on the binding of substrate and AMP even after extensive blocking of SH groups by iodoacetamide. On the other hand, the present experiments as well as those by Gold (1968) and by Damjanovich *et al.* (1967) show that blocking of the highly reactive sulfhydryl groups increases K_{AMP} significantly while $K_{glucose\text{-}1\text{-}P}$ is unaffected. This indicates an interaction between the highly reactive SH groups and the binding site of AMP.

The interaction between the SH groups and the binding site of AMP is even more convincingly demonstrated in the experiments showing that the presence of AMP increases the reactivity of the slowly reacting subgroup of the highly reactive SH groups by a factor of nearly 2. The fact that the presence of glucose-1-P decreases the reactivity of the SH groups demonstrates that an interaction occurs also between these sites. Birkett *et al.* (1971) have previously found that when the highly reactive SH groups are blocked by a fluorescent label, the fluorescence is partially quenched upon addition of AMP and glucose-1-P. The present results demonstrate, however, that AMP and glucose-1-P affect the SH groups in different ways. Previous kinetic experiments have demonstrated that glucose-1-P increases the binding of AMP. The present results demostrate that the effect of AMP on the sulfhydryl groups is enhanced by the presence of glucose-1-P while ATP, which is an inhibitor of the enzyme, abolished the enhancement by glucose-1-P.

Attempts to define conformational states of phosphorylase *b* from studies of optical rotatory dispersion and circular dichroism have been unsuccessful (Hedrick, 1966; Johnson and Graves, 1966). On the other hand Bresler and Firsov (1968) as well as Buc and Buc (1968) have observed spectral changes occurring in the presence of substrate and the allosteric activator. It has been claimed that this may represent the transition of the enzyme from T to R state. This transition induced by AMP has been studied in further detail by Kastenschmidt *et al.* (1968b). These authors claim that state T does not bind AMP while R binds AMP tightly. The present results indicate that the two subclasses of highly reactive SH groups reflect the different reactivities of the same SH groups when the enzyme is present in the different states. It is of interest to note that there seems to be no continuous change from the fast to the slowly reacting SH groups, thus the enzyme is either present in the one or in the other state.

Acknowledgment

Phosphorylase *b* was kindly supplied by the Department of Biophysics, Medical University of Debrecen, Hungary.

References

Appleman, M. M., Yunis, A. A., Krebs, E. G., and Fischer, E. H. (1963), *J. Biol. Chem. 238,* 1358.

Avramovic-Zikic, O., Smillie, L. B., and Madsen, N. B. (1970), *J. Biol. Chem. 245,* 1558.

Battell, M. L., Zarkadas, C. G., Smillie, L. B., and Madsen, N. B. (1968), *J. Biol. Chem. 243,* 6202.

Birkett, D. J., Dwek, R. A., Radda, G. K., Richards, R. E., and Salmon, A. G. (1971), *Eur. J. Biochem. 20,* 494.

Bresler, S., and Firsov, L. (1968), *J. Mol. Biol. 35,* 131.

Buc, M. H., and Buc, H. (1968), *in* Regulation of Enzyme Activity and Allosteric Interactions, Kvamme, E., and Pihl, A., Ed., Oslo, Universitetsforlaget, p 109.

Damjanovich, S., and Kleppe, K. (1966), *Biochim. Biophys. Acta 122,* 145.

Damjanovich, S., Sanner, T., and Pihl, A. (1967), *Eur. J. Biochem. 1,* 347.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82,* 70.

Fischer, E. H., and Krebs, E. G. (1958), *J. Biol. Chem. 231,* 65.

Gold, A. M. (1968), *Biochemistry 7,* 2106.

Gold, A. M. and Blackman, D. (1970), *Biochemistry 9,* 4480.

Hasinoff, B. B., Madsen, N. B., and Avramovic-Zikic, O. (1971), *Can. J. Biochem. 49,* 742.

Hedrick, J. L. (1966), *Arch. Biochem. Biophys. 114,* 216.

Johnson, G. F., and Graves, D. F. (1966), *Biochemistry 5,* 2906.

Jokai, I., Damjanovich, S., and Toth, S. (1965), *Arch. Biochem. Biophys. 112,* 471.

Kastenschmidt, L. L., Kastenschmidt, J., and Helmreich, E. (1968a), *Biochemistry 7,* 3590.

Kastenschmidt, L. L., Kastenschmidt, J., and Helmreich, E.

(1968b), *Biochemistry 7,* 4543.
Kleppe, K., and Damjanovich, S. (1969), *Biochim. Biophys. Acta 185,* 88.
Madsen, N. B., and Cori, C. F. (1956), *J. Biol. Chem. 223,* 1055.
Sanner, T. (1971), *Biochim. Biophys. Acta 250,* 297.
Seery, V. L., Fischer, E. H., and Teller, D. C. (1967), *Biochemistry 6,* 3315.
Taussky, H. H., and Shorr, E. (1953), *J. Biol. Chem. 202,* 675.
Zarkadas, C. G., Smillie, L. B., and Madsen, N. B. (1970), *Can. J. Biochem. 48,* 763.

The Quaternary Structure of Citrate Synthase from *Escherichia coli* K12†

E. K. Tong and Harry W. Duckworth*

ABSTRACT: A combination of equilibrium ultracentrifugation and polyacrylamide gel electrophoresis techniques has been used to establish the quaternary structure of citrate synthase from acetate-grown *Escherichia coli* K12 3000. In polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS), the pure enzyme showed one major band whose mobility was consistent with a molecular weight of 46,000 ± 2000 g/mol, and a little material of 87,000 ± 5000 g/mol. When first cross-linked with dimethyl suberimidate and then submitted to electrophoresis in SDS, citrate synthase showed six bands, in widely different amounts, whose apparent molecular weights were almost integral multiples of 47,000 g/mol. The dimer was the major product of the cross-linking procedure. In 6 M guanidine HCl at pH 7.0, citrate synthase behaved as a single component in high-speed sedimentation equilibrium experiments, with a weight average molecular weight of 43,400 ± 300 g/mol. The molecular weight of native citrate synthase was investigated by high-speed sedimentation equilibrium ultracentrifugation under different conditions of pH and KCl concentration. In 0.02 M Tris-Cl at pH 7.0 and 7.8, the enzyme was a mixture of oligomers, with species ranging from monomer (47,000 g/mol) to greater than decamer being present. At pH 9.0, only dimer was seen (94,000 g/mol). Large aggregates were present at pH 10.0. The addition of small amounts of KCl, a potent activator of the enzyme, simplified the mixture of oligomers considerably at pH 7.8. A detailed analysis of the data with 0.05 M KCl indicated that dimer and hexamer were the only species present, with marked nonideality. Increasing the KCl concentration to 0.10 M converted all the enzyme to hexamer. The amino acid composition of *E. coli* citrate synthase was presented. Taken together with peptide mapping experiments of others (J. A. Wright and B. D. Sanwal (1971), *J. Biol. Chem. 246* 1689), it indicates that the subunits have all the same or very similar amino acid sequences. The dansylation method revealed only methionine at the N-termini of the citrate synthase polypeptide chains. Citrate synthase from *E. coli* thus resembles the enzyme from eukaryotes in that it consists of subunits weighing just under 50,000 g/mol, although these subunits are more highly aggregated in the bacterial enzyme under most conditions. This conclusion is in disagreement with that of Wright and Sanwal (1971, see above), who reported a subunit size of 62,000 g/mol.

Citrate synthase catalyzes the reaction by which acetyl-CoA carbon enters the tricarboxylic acid cycle. The enzyme has been studied from a number of sources, and is often found to exhibit allosteric properties (Srere, 1972). The eukaryotic enzymes have molecular weights near 100,000 g/mol, and consist of two apparently identical subunits (Srere, 1972). Many bacterial enzymes are much larger (Weitzman and Dunmore, 1969), but there is little information about their subunit structures. Wright and Sanwal (1971) have reported that *Escherichia coli* citrate synthase is a mixture of octamers and tetramers of subunits the molecular weight of which is about 62,000 g/mol. Some of their results have been confirmed by Danson and Weitzman (1973). Our interest in the allosteric properties of the *E. coli* enzyme has prompted us to investigate its subunit structure in more detail. The results are presented in this paper, and they suggest that the molecule as isolated from *E. coli* K12 3000, grown on acetate, consists of subunits of size about 47,000 g/mol, in a state of aggregation which varies with pH and KCl concentration.

Experimental Section

Enzyme. Citrate synthase was purified from *Escherichia coli* K12 strain 3000 by a method like those published by Faloona and Srere (1969) and Wright and Sanwal (1971). We omitted the heat step used by the first group, and the reverse ammonium sulfate step of the second group, since neither step seemed to affect the final purity of the protein as judged by polyacrylamide gel electrophoresis, at pH 8.9 by the method of Davis (1964). Our citrate synthase shows three protein bands upon gel electrophoresis under these conditions, as did the preparations of the groups just cited. Various indications that our preparations are essentially homogeneous emerge in the Results section of this paper.

† From the Department of Chemistry, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2. *Received August 26, 1974.* Supported by the National Research Council of Canada and by the Research Board of the University of Manitoba. Taken in part from a thesis submitted by E.K.T. to the Faculty of Graduate Studies of the University of Manitoba, in partial fulfillment of the requirements for the degree Master of Science.